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SCIENCE AND PRACTICE

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Production and characterization of chimeric Bst-like polymerases and their application in isothermal amplification combined with rapid RNA extraction methods using the example of the mumps virus

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Abstract

Introduction. Bst polymerase plays a key role in the rapid diagnosis of infectious diseases due to its unique biochemical properties and potential application in loop-mediated isothermal amplification (LAMP). Several analogs of Bst polymerase have been described in the literature; however, these enzymes have not been widely used in molecular diagnostics.

The aim of the study is to obtain recombinant Bst and Btlv polymerases with the Sso7d domain and to test new possibilities for their application.

Materials and methods. Expression constructs carrying the polymerase gene were obtained using standard genetic engineering methods. The target enzyme was produced in *Escherichia coli* cells. Purification was carried out using metal-affinity chromatography methods followed by dialysis and concentration. RNA-dependent DNA polymerase (reverse transcriptase) and DNA polymerase activities of the enzymes were determined using non-radioactive methods with fluorescent detection. The functional properties of the enzymes were assessed using the Amplisens SARS-CoV-2-IT reagent kit and a method designed for the detection of mumps virus RNA in biological material using the LAMP format combined with reverse transcription.

Results. In the *E. coli*-based expression system, the following recombinant chimeric enzymes with displacing activity have been obtained: Bst_Sso7d, Bst_Sso7d_mut4 and Btlv_Sso7d. The developed cultivation and purification protocols allow for the production of enzymes in soluble form with a yield of up to 25% of the collected cell mass. Functional testing showed that in LAMP, the chimeric polymerases demonstrated similar activity to Bst polymerase without the Sso7d domain. At the same time, the Btlv_Sso7d polymerase exhibited increased reverse transcriptase activity and resistance to inhibitors.

Conclusion. The obtained chimeric polymerase Btlv_Sso7d, due to its improved properties, can be used in reagent kits for the diagnosis of infectious diseases by the LAMP method when using nucleic acid extraction methods.

Keywords: *Bst polymerase, displacing activity, isothermal amplification, inhibitor resistance*

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Оригинальное исследование
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Получение и характеристика химерных Bst-подобных полимераз и их применение в изотермической амплификации в сочетании с экспресс-методами выделения РНК на примере вируса эпидемического паротита

Замотаева Т.Л.✉, Дедяева Е.А., Михеева О.О., Пика М.И., Черкашин Е.А., Черкашина А.С., Акимкин В.Г.

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Аннотация

Введение. Bst-полимераза играет ключевую роль в экспресс-диагностике инфекционных заболеваний благодаря своим уникальным биохимическим свойствам и возможности применения в петлевой изотермической амплификации (LAMP). В литературе описано несколько аналогов Bst-полимеразы, однако данные ферменты не получили широкого применения в молекулярной диагностике.

Цель работы — получение рекомбинантных Bst- и Btlv-полимераз с Sso7d-доменом и тестирование новых возможностей для их применения.

Материалы и методы. Экспрессионные конструкции, несущие ген полимеразы, получали стандартными методами генетической инженерии. Целевой фермент был наработан в клетках *Escherichia coli.* Очистку проводили методами металл-аффинной хроматографии с последующим диализом и концентрированием. РНК-зависимую ДНК-полимеразную (ревертазную) и ДНК-полимеразную активности ферментов определяли с помощью нерадиоактивных методик с флуоресцентной детекцией. Функциональные свойства ферментов оценивали в наборе реагентов «АмплиСенс SARS-CoV-2-IT» и в методике, предназначенной для определения в биологическом материале РНК вируса эпидемического паротита в формате LAMP, совмещённой с обратной транскрипцией.

Результаты. В системе экспрессии на основе клеток *E. coli* получены рекомбинантные химерные ферменты с вытесняющей активностью: Bst_Sso7d, Bst_Sso7d_mut4 и Btlv_Sso7d. Разработанные протоколы культивирования и очистки позволяют получать ферменты в растворимой форме с выходом до 25% от собранной клеточной массы. Функциональное тестирование показало, что в LAMP химерные полимеразы демонстрировали сходную активность с Bst-полимеразой без Sso7d-домена. Вместе с тем полимераза Btlv_Sso7d имела повышенную ревертазную активность и устойчивость к ингибиторам.

Заключение. Полученная химерная полимераза Btlv_Sso7d, благодаря своим улучшенным свойствам, может быть использована в наборах реагентов для диагностики инфекционных заболеваний методом LAMP при использовании методов экспресс-экстракции нуклеиновых кислот.

Ключевые слова: *Bst-полимераза, вытесняющая активность, изотермическая амплификация, устойчивость к ингибиторам*

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Introduction

Reducing the time of research with the help of rapid tests is one of the key trends in laboratory diagnostics. This approach is particularly relevant for increasing the throughput of the laboratory in the context of mass screenings and allows medical personnel or epidemiologists to perform diagnostics in resource-limited settings, which contributes to providing timely assistance to patients, quickly identifying infected individuals, promptly investigating infectious outbreaks, and taking appropriate epidemiological measures, as well as preventing the excessive prescription of preventive and therapeutic measures. Such rapid tests must meet certain requirements such as high accuracy and speed, simplicity and accessibility, as well as high stability during storage and transportation [1, 2].

The coronavirus pandemic has spurred the development of isothermal amplification methods. The loop-mediated isothermal amplification (LAMP) method [3–5] has high sensitivity and specificity, and an important advantage is that the amplification reaction occurs at a constant temperature (without thermal cycling). This allows for research to be conducted both in equipped clinical diagnostic laboratories and in field conditions where specialized laboratory equipment is not available. The key role in the further development of reagent kits for the express diagnosis of infectious diseases using the LAMP method belongs to Bst polymerase [6, 7] and its modifications. Bst polymerase is a large fragment of DNA polymerase I (Bst-LF), isolated from the *Geobacillus stearothermophilus* thermophilic bacterium (formerly known as *Bacillus stearothermophilus*) [8] and having an optimal temperature of 60–70°C. The enzyme was isolated by J. Stenesh et al. in 1972 [9], four years before the discovery of Taq polymerase [10, 11]. Subsequently, both for research purposes and in production solutions, recombinant enzymes, particularly Bst polymerase, cloned and expressed in *Escherichia coli* bacterial cells, have generally been used. The bacterial system based on *E. coli* cells is characterized by simplicity and low cultivation costs, high microbial growth rates, and a wide range of different vectors for recombinant protein expression have been developed for it. Approaches to cloning and obtaining recombinant enzymes in a bacterial expression system based on *E. coli* cells have been described for Bst polymerase and similar polymerases with displacing activity from other organisms [12–14]. Since the specific catalytic activity of the enzyme is influenced, among other factors, by the characteristics of the nucleotide sequence of the gene, expression conditions, as well as the extraction and purification protocol, all these stages require optimization when obtaining any enzyme.

The main approaches to modifying the physicochemical characteristics of an enzyme according to the practical objectives of the user are directed mutagenesis and the addition of protein domains with specified properties [15, 16]. Such modifications allow for an increased yield of soluble enzyme during expression in *E. coli* cells, simplify the purification process, and also produce enzymes with improved properties such as higher activity and thermostability, as well as resistance to salts and inhibitors. The unmodified Bst polymerase has insufficient processivity because, in the native organism, it is primarily involved in DNA repair [17]. The modified enzyme exhibits much greater processivity, which is due to the presence of auxiliary proteins in the cells that enhance the stability of the polymerase-DNA complex. The Sso7d protein belongs to the family of DNA-binding proteins isolated from the *Sulfolobus* genus archaeon, and is stable over a wide range of temperatures and pH. Several authors describe a strategy of fusing polymerases with the Sso7d protein or similar proteins (Sto7d, SSB, TBD, DBD) to obtain chimeric enzymes with increased processivity, displacing activity, thermostability and tolerance to inhibitors, including urea, whole blood and NaCl [18–22].

Thermophilic bacteria of related species can develop different survival strategies, which are due, among other things, to differences in the properties of their enzymes. In this regard, potentially interesting directions include the cloning and obtaining of recombinant DNA polymerases from new sources, such as a related organism and the closest homolog — the *Geobacillus thermoleovorans* thermophilic bacterium (formerly known as *Bacillus thermoleovorans*) [23, 24].

The aim of this study was to obtain chimeric Bst-like polymerases from *G. stearothermophilus* and to compare them with the homolog Btlv polymerase from *G. thermoleovorans* to evaluate their potential application in isothermal amplification reactions combined with rapid RNA extraction methods.

Materials and methods

Obtaining the Bst_Sso7d, Bst_Sso7d_mut4, Btlv_Sso7d genes

The nucleotide sequence encoding the amino acid sequence of Btlv polymerase from *G. thermoleovorans* was obtained by the assembly method using long overlapping primers—the staircase method [25]. Restriction sites were introduced at the ends of the nucleotide sequence: NdeI at the 5' end and XhoI at the 3' end for subsequent re-cloning into the pET16b+ expression vector. As a result, the pET16-Btlv-Nhis expression vector was obtained. The correctness of the nucleotide sequence of the cloned gene was confirmed by sequencing.

As sources of the *Bst* and *Bst_mut4* genes, plasmids previously obtained in the laboratory were used: pET16_Bst and pET16_Bst_NHis_m4, respectively [7, 26]. The template for amplifying the *Sso7d* gene was also the previously obtained construct pPSS, which contains the wild-type *Sso7d* gene.

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Table 1. Primer sequences used for gene cloning

Matrix	Name	5'-3' sequence	PCR2
		Bst_Sso7d	
pET16_Bst	BstF	gaaaggaggaggagctctaacatctgcggaaggcgaaaaaccg	
	BstR	agtctcgagttatttcgcatcataccagg	v
pPSS	SsoF	tcgtcatatggcgaccgtgaagttcaagtataaag	v
	SsoR	agatgttagagctcctcctcctttcttctgtttttccag	
		Bst_Sso7d_mut4	
pET16_Bst_NHis_m4	BstF	gaaaggaggaggagctctaacatctgcggaaggcgaaaaaccg	
	BstR	agtctcgagttatttcgcatcataccagg	v
pPSS	SsoF	tcgtcatatggcgaccgtgaagttcaagtataaag	v
	SsoR	agatgttagagctcctcctcctttcttctgtttttccag	
		Btlv_Sso7d	
pET16-Btlv-Nhis	BtlvF	gaaaggaggaggagctctaacatctccgtcttctgaggaagaaaagcc	
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pPSS	SsoF	tcgtcatatggcgaccgtgaagttcaagtataaag	v
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To obtain the genes of chimeric enzymes, amplification of the target enzyme gene and the *Sso7d* gene of the DNA-binding domain was carried out. The obtained amplicons were extracted and purified from the gel and ligated together using flanking primers (**Table 1**).

To obtain protruding A-ends, the purified amplicon was incubated for 30 minutes at 72°C in the presence of Taq polymerase and a mixture of deoxynucleotides (dNTPs). The target product was then cloned into the pGEM-T vector (Promega). The presence of the target sequence and its accuracy were confirmed by Sanger sequencing.

Obtaining expression plasmid vectors containing the Bst-Sso7d, Bst-mut4-Sso7d, Btlv-Sso7d genes

Plasmid DNA containing the gene of the chimeric enzyme was treated with the NdeI and XhoI restriction endonucleases, and the resulting restriction product was cloned into the pET16b+ plasmid vector, which had been pre-treated with the same restriction endonucleases. As a result, expression vectors containing genes that encode the following hybrid proteins were obtained: Bst_Sso7d with a molecular weight of 75 kDa, Bst_Sso7d_mut4 with a molecular weight of 75.2 kDa, and Btlv_Sso7d with a molecular weight of 75 kDa. The accuracy of the nucleotide sequence of the cloned genes was confirmed by sequencing.

Selection of E. coli strains for the expression of the Bst_Sso7d, Bst_Sso7d_mut4, and Btlv_Sso7d genes

As host strains for the constructed expression vectors pET16-Bst_Sso7d, pET16-Bst_Sso7d_mut4, and pET16-Btlv_Sso7d, the *E. coli* strains ER2566, BL21de3 pLys, and Rosetta De3 were used. Transformed cells were plated on LB medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) with agar containing 100 μg/mL ampicillin for ER2566 cells and 20 μg/mL chloramphenicol for BL21 (DE3) pLys and Rosetta (DE3) cells, and incubated for 16 hours at 37°C to obtain individual colonies. Then, 7–8 colonies were transferred to 100 mL of LB medium with 100 μg/mL ampicillin and incubated for 18 hours at 37°C on a shaker at 180 rpm to obtain an overnight culture. The obtained overnight cultures of *E. coli* producer strains were transferred to LB medium with 100 μg/mL of ampicillin in Erlenmeyer flasks (the inoculation percentage was 2%) and incubated at 37°C with shaking at 160 rpm. When the optical density of the bacterial culture reached 0.8 optical units, isopropyl-β-D1-thiogalactopyranoside was added to a concentration of 0.4 mM, and the culture was incubated at 23°C and 37°C for 4 and 24 hours. The optical density was measured spectrophotometrically at a wavelength of 595 nm. Cell biomass was obtained by centrifugation for 20 minutes at 4°C and 4000 rpm on an Avanti JXN-30 (Beckman Coulter) centrifuge.

Isolation of Bst_Sso7d, Bst_Sso7d_mut4, and Btlv_Sso7d

The cell biomass (2 g) of *E. coli* BL21 (DE3)pLys/pET16-Bst_Sso7d, BL21 (DE3)pLys/pET16-Bst_Sso7d_mut4, and BL21 (DE3)pLys/pET16-Btlv_Sso7d producer strains was re-suspended in a buffer solution of 50 mM Tris-HCl, 100 mM NaCl, pH 8.5 with 1 mM PMSF at a ratio of 1:10 (w/v) and disrupted using a Branson sonifier 250 ultrasonic disintegrator (Branson Ultrasonics) for 20 minutes at 4°C (cycle — 0.5 s, amplitude — 50%). Then centrifuged at 8000 rpm for 30 minutes in an Allegra X-30R centrifuge (Beckman Coulter). After centrifugation, the supernatant was di-

luted 2-fold with a buffer solution of 50 mM Tris-HCl, 100 mM NaCl pH 8.5 and applied to the IMAC FF chromatographic sorbent, which had been pre-equilibrated with a buffer solution of 50 mM Tris-HCl, 100 mM NaCl pH 8.5 (buffer solution A). The removal of contaminating proteins was carried out with buffer solution A. The target protein was eluted with a linear gradient of buffer solution A with 500 mM imidazole.

After metal-chelate chromatography, the fractions containing the target protein were dialyzed against a buffer solution of 20 mM Tris, 100 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA, pH 8.3. After dialysis, glycerol was added to the protein solution to a final concentration of 50%.

Determination of enzyme activity

The RNA-dependent DNA polymerase (reverse transcriptase) activity of the enzymes was determined using a non-radioactive method with fluorescent detection, based on the formation of a duplex of polyadenylated RNA and the oligonucleotide primer dT18, into which the GelStar intercalating dye (Lonza) was incorporated. The DNA polymerase activity of the enzymes was determined using a non-radioactive method [27]. An oligonucleotide containing a hairpin structure at the 3' end was used as the template. In the presence of Mg^{2+} ions, polymerases catalyzed the incorporation of deoxynucleotides, thereby extending the matrix. The described non-radioactive methods allow for the determination of enzyme activities using amplifiers with an optical module for real-time fluorescence detection.

The stability of the enzymes to temperature exposure was assessed using differential scanning fluorimetry in the range of 55–85°C at 1°C increments, with a step duration of 50 seconds. The melting curves were detected in the Fam channel on the CFX 96 device (Bio-Rad Laboratories).

The optimal ion concentrations in the reaction mixture were selected similarly to the analysis of polymerase activity using KCl concentrations in the range of 50–500 mM.

Analytical methods

The protein concentration was determined using the QuDye Protein kit (LLC LumiProbe RUS) on the Qubit 4 fluorimeter (Thermo Scientific), and the protein purity was assessed using SDS-PAGE electrophoresis under denaturing conditions [28]. When performing SDS-PAGE electrophoresis under denaturing conditions, a protein length marker — a molecular weight marker (ThermoScientific) — was used.

Loop-mediated isothermal amplification combined with reverse transcription

To assess the potential use of the obtained chimeric enzymes in new reagent kits for the diagnosis of infectious diseases, testing was conducted on the AmpliSens SARS-CoV-2-IT reagent kit (RU No. FSS 2021/14599). As samples, a genetic construct representing the MS2 bacteriophage with a specific insertion of the coronavirus ORF1ab gene was used. The reaction mixtures contained 0.8 μL of the tested enzymes at a concentration of 0.288 U/μL or the same amount of Bst polymerase without the Sso7d domain, as well as Reverse Transcriptase (MMLv). Each sample was amplified in 3 replicates on the Rotor-Gene Q device (Qiagen).

The functional properties of the enzymes were also tested in a laboratory method designed for detecting the RNA of the mumps virus in biological material using the LAMP format with combined reverse transcription. We amplified a fragment of the phosphoprotein gene of the mumps virus (amplicon size 225 base pairs, GC content 54%). Urine and oropharyngeal mucosal swabs were used as samples, and the presence of mumps virus RNA was confirmed by Sanger sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on the 3500xL Genetic Analyzer (Thermo Fisher Scientific).

Nucleic acid extraction was performed using the EDEM express kit (RU No. 2010/07828), as well as a lysis solution containing guanidine hydrochloride. As a control extraction method, the RIBO-prep reagent kit (RU No. FSS 2008/03147) was used. The reaction mixtures contained oligonucleotides complementary to the target amplification sites, as well as an intercalating dye, which allowed for the registration of the accumulation of the specific amplification product by measuring the intensity of the fluorescent signal in real-time mode; as well as a mixture of glycerin with thioglycerol, salts, and surfactants (all additional reagents used were developed and produced at the Central Research Institute of Epidemiology of Rospotrebnadzor). As a control batch, the non-mutated form of Bst polymerase without the Sso7d domain was used at a protein concentration equal to that of the tested enzymes. The reaction was conducted according to the program: 37°C for 5 minutes, 65°C for 30 seconds, 40 cycles with detection in the FAM channel (total duration 25 minutes).

Assessment of enzyme stability to LAMP inhibitors

To assess the stability of LAMP enzymes against inhibitors, mucin (Sigma-Aldrich) was used, as well as pre-characterized residual samples of human biological material (urine, pharyngeal mucosa swabs, plasma, and whole blood). Swabs from the oropharyngeal mucosa were stored in a transport medium for the storage and transport of respiratory swabs (RU No. FSS 2009/05011; Central Research Institute of Epidemiology of Rospotrebnadzor).

Inhibitors were added to the reaction mixture for LAMP. The reaction was conducted according to the program: 37°C for 5 minutes, 65°C for 30 seconds, 40 cycles with detection in the FAM channel (total

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duration 25 minutes). The results were evaluated based on the presence/absence of the fluorescent signal (detected/not detected) and the values of the threshold cycles (Ct), which is sufficient for diagnostic systems with qualitative determination.

Results

The Sso7d family includes small, numerous, non-specific DNA-binding proteins, first discovered in the *Archaea sulfolobus* hyperthermophilic bacterium. They have a mass of 7–10 kDa and exhibit various types of functional activity: stabilization of the double helix, annealing of DNA at temperatures above its melting point, and prevention of protein aggregation. Moreover, such proteins alter the conformation of DNA, causing the unwinding of the DNA double helix [29].

To obtain recombinant Bst_mut4- and Btlv-polymerases, unique synthetic sequences were developed with consideration of codon usage optimization for expression in *E. coli*. Next, the *Sso7d* gene was attached to the Bst- and Btlv-polymerase genes, and these constructs were cloned into the pET16b+expression vector for prokaryotic expression in *E. coli* cells. During the selection of host strains for the expression of the genes *Bst_Sso7d, Bst_Sso7d_mut4* and *Btlv_Sso7d*, the dynamics of enzyme accumulation were studied at different temperatures (23 and 37°C) and induction times for protein biosynthesis (4 and 24 hours). As a result, it was shown that all enzymes were effectively accumulated in a soluble form at 23°C for 24 hours in *E. coli* BL21 (DE3)pLys cells (**Tables 2–4**).

For the purification of enzymes from cellular proteins, metal-affinity chromatography with a linear imidazole gradient was used (**Fig. 1**).

Fractions with a purity of over 90% were pooled and dialyzed against a buffer solution of 20 mM Tris, 100 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA pH 8.3, followed by concentration. According to the electrophoretic analysis, the enzyme purity was at least 90% (**Fig. 2**) with a concentration of at least 2 mg/mL.

It is worth noting that, despite the higher percentage of protein content from the total mass of wet cells: in the case of Bst_Sso7d expression — 25% compared to 23% for Bst_Sso7d_mut4 and Btlv_Sso7d, after purification, the latter two enzymes showed higher yields. This is due to the fact that optimizing the codon composition of the coding sequence leads to an increase in the yield of the protein in a soluble form.

Testing the polymerase activity of the isolated enzymes Bst_Sso7d, Bst_Sso7d_mut4, and Btlv_Sso7d was conducted on samples of MS2 bacteriophage RNA with a specific insert containing a fragment of the SARS-CoV-2 genome, using the reverse transcrip-

Table 2. Selection of host strains for the expression of *Bst_Sso7d* genes

E-coli host strain	Cultivation conditions	Protein content, %	Cell biomass extraction, g/L
ER2566	23°C, 4 h	10	2,1
	23°C, 24 h	15	2,3
	37°C, 4 h	12	2,3
	37°C, 24 h	12	2,5
Rosetta De3	23°C, 4 h	23	2,4
	23°C, 24 h	23	2,6
	37°C, 4 h	21	2,5
	37°C, 24 h	24	2,9
BL21 De3 pLys	23°C, 4 h	23	2,7
	23°C, 24 h	**25**	**3,14**
	37°C, 4 h	24	2,9
	37°C, 24 h	21	3,2

Table 3. Selection of host strains for the expression of *Bst_Sso7d_mut4* genes

E-coli host strain	Cultivation conditions	Protein content, %	Cell biomass extraction, g/L
ER2566	23°C, 4 h	10	2,1
	23°C, 24 h	11	2,5
	37°C, 4 h	14	2,5
	37°C, 24 h	16	1,7
Rosetta De3	23°C, 4 h	19	2,2
	23°C, 24 h	21	2,4
	37°C, 4 h	21	2,3
	37°C, 24 h	22	2,5
BL21 De3 pLys	23°C, 4 h	21	2,1
	23°C, 24 h	**23**	**2,6**
	37°C, 4 h	21	2,5
	37°C, 24 h	20	2,7

Table 4. Selection of host strains for the expression of *Btlv_Sso7d* genes

E-coli host strain	Cultivation conditions	Protein content, %	Cell biomass extraction, g/L
ER2566	23°C, 4 h	18	2,0
	23°C, 24 h	16	2,5
	37°C, 4 h	16	2,4
	37°C, 24 h	14	2,8
Rosetta De3	23°C, 4 h	18	2,3
	23°C, 24 h	17	2,4
	37°C, 4 h	19	2,5
	37°C, 24 h	19	2,7
BL21 De3 pLys	23°C, 4 h	21	2,4
	23°C, 24 h	**23**	**2,5**
	37°C, 4 h	23	2,5
	37°C, 24 h	21	2,7

tion LAMP method with reagents from the AmpliSens SARS-CoV-2-IT kit. The concentration of the template was 10^5 copies per reaction. All new enzymes exhibited polymerase activity; however, the average threshold cycle values differed from the control batch of Bst polymerase without the Sso7d domain, with an increase in values by an average of 3.73 for Bst_Sso7d, 3.42 for Bst_Sso7d_mut4 and 2.88 for Btlv_Sso7d (**Fig. 3**), which may indicate suboptimal reaction conditions for the new enzymes.

All the studied enzymes showed comparable results in experiments assessing their resistance to temperature exposure (**Fig. 4**).

The isolated enzymes Bst_Sso7d, Bst_Sso7d_mut4, and the comparison enzyme exhibited weak RNA-dependent DNA polymerase (reverse transcriptase) activity both in the method [7] and when tested in the AmpliSens *SARS-CoV-2*-IT kit without the reverse transcriptase enzyme. At the same time, Btlv_Sso7d polymerase exhibited reverse transcriptase activity comparable to that of reverse transcriptase (MMLv), but only in the presence of KCl and $(NH_4)_2SO_4$, which allows the enzyme to be used not only for DNA/cDNA amplification but also for RNA reverse transcription. Changing the composition of the reaction mixture (mainly the addition of KCl) allowed for an increase

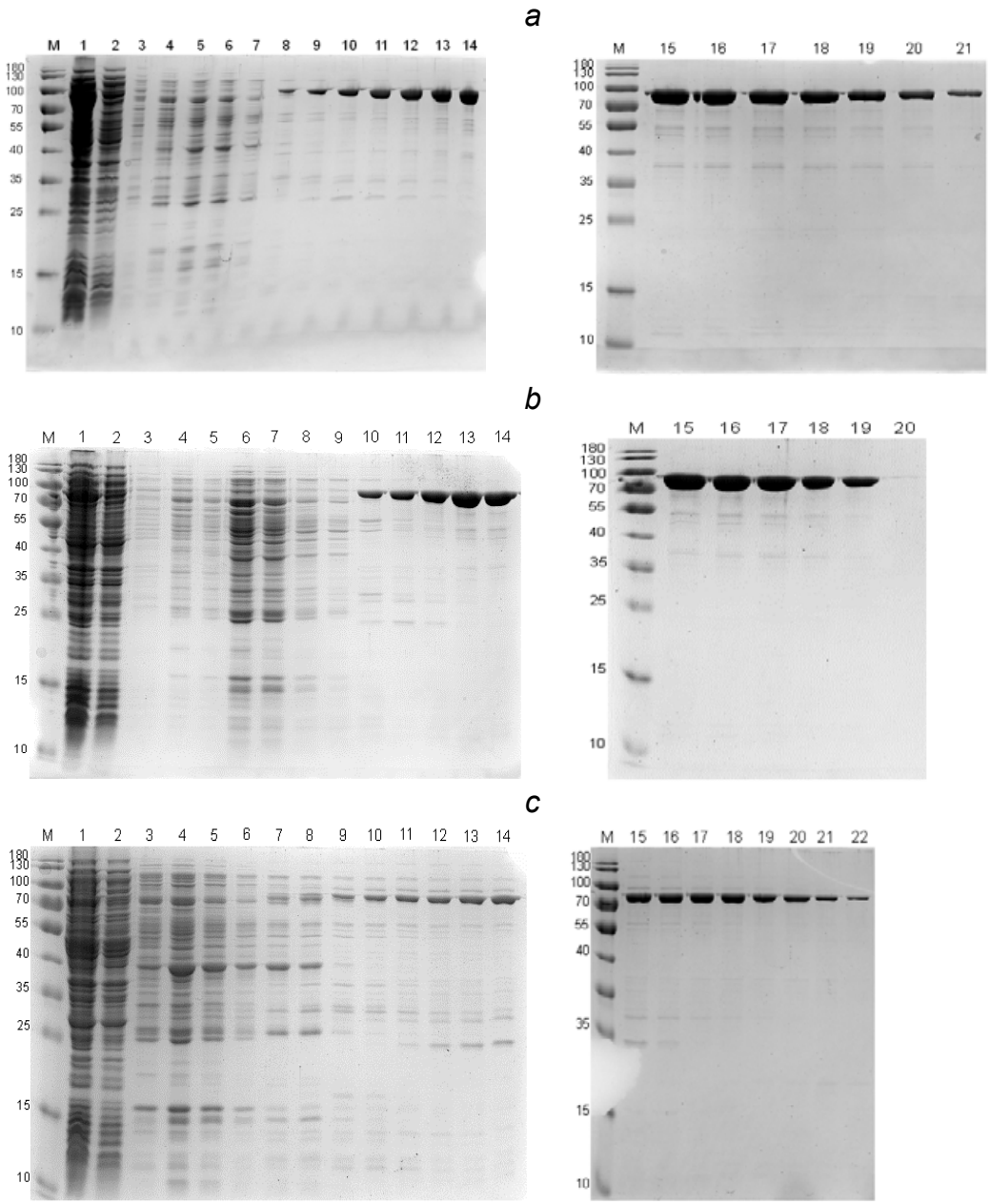

Fig. 1. Electrophoregram of purified enzymes Bst_Sso7d (*a*), Bst_Sso7d_mut4 (*b*), and Btlv_Sso7d (*c*).
M — molecular weight marker; 1 — clarified cell lysate; 2 — wash with metal-chelating resin; subsequent — fractions after affinity chromatography.

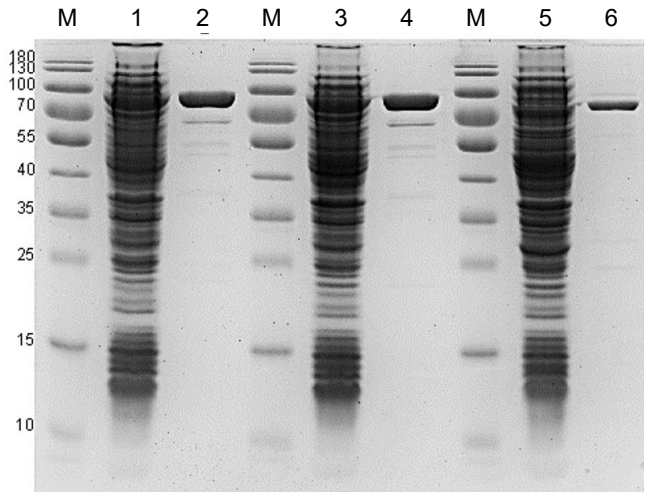

Fig. 2. Electropherogram of the purification of enzymes Bst_Sso7d_mut4, Bst_Sso7d, and Btlv_Sso7d.

M — molecular weight marker; 1, 3, 5 — clarified cell lysate of Bst_ Sso7d_mut4, Bst_ Sso7d, and Btlv_Sso7d respectively; 2, 4, 6 — final purified enzyme preparations of Bst_Sso7d_mut4, Bst_ Sso7d, and Btlv_Sso7d respectively.

in the polymerase activity of all chimeric enzymes, with the optimal concentration of KCl in the buffer for LAMP being 200 mM (**Fig. 5**).

LAMP-based reagent kits allow for the reduction of amplification time from 1.5–2.5 hours (PCR method) to 25–40 minutes, while maintaining high specificity due to the use of 4–6 oligonucleotides. However, the process of nucleic acid extraction from biological material is still quite lengthy. The samples under investigation are treated with a lysis solution, resulting in the destruction of bacterial cell walls and viral envelopes, followed by the release of cellular components and nucleic acids into the solution. The subsequent wash steps in the extraction protocol allow for the removal of substances and cell components from the solution that inhibit PCR. Express methods are characterized by the absence of a washing step or the presence of only one washing step: clinical material taken in a special transport medium (for example, TS-EDEM) is subjected to thermal treatment and centrifugation, resulting in the precipitation of insoluble components, while the nucleic acids remaining in the supernatant are used for PCR. However, after disinfection without extraction or after express extraction, RNA/DNA samples contain impurities that can act as PCR inhibitors, such as components of transport media, lysis buffer, blood, swabs, urine and other biomaterials.

The functional properties of the polymerases obtained in the study were tested for resistance to the most commonly encountered inhibitors (components of whole blood, plasma, urine, mucin) in the LAMP reaction in a model system using positive control samples. Inhibitors were added to the reaction mixture containing primers from the AmpliSens SARS-CoV-2-IT reagent kit and isolated MS2 bacteriophage RNA with a specific insert containing a fragment of the SARS-CoV-2 genome. The results were evaluated based on the presence/absence of the fluorescent signal (detected/not detected), which is sufficient for diagnostic systems with qualitative determination (**Table 5**).

Modified enzymes based on Bst polymerase, containing the DNA-binding domain Sso7D: Bst_Sso7d and Bst_Sso7d_mut4, demonstrated reduced stability in the presence of whole blood and urine in the reaction mixture compared to the control enzyme. At the same time, the Btlv_Sso7d enzyme is characterized by higher resistance to the presence of whole blood (10 vol. %), plasma (1 vol. %), and urine (10 vol. %). All enzymes retain their activity in the presence of mucin up to 0.5 mg/ml.

Further experiments to determine the effect of inhibitors on the efficiency of the LAMP reaction were

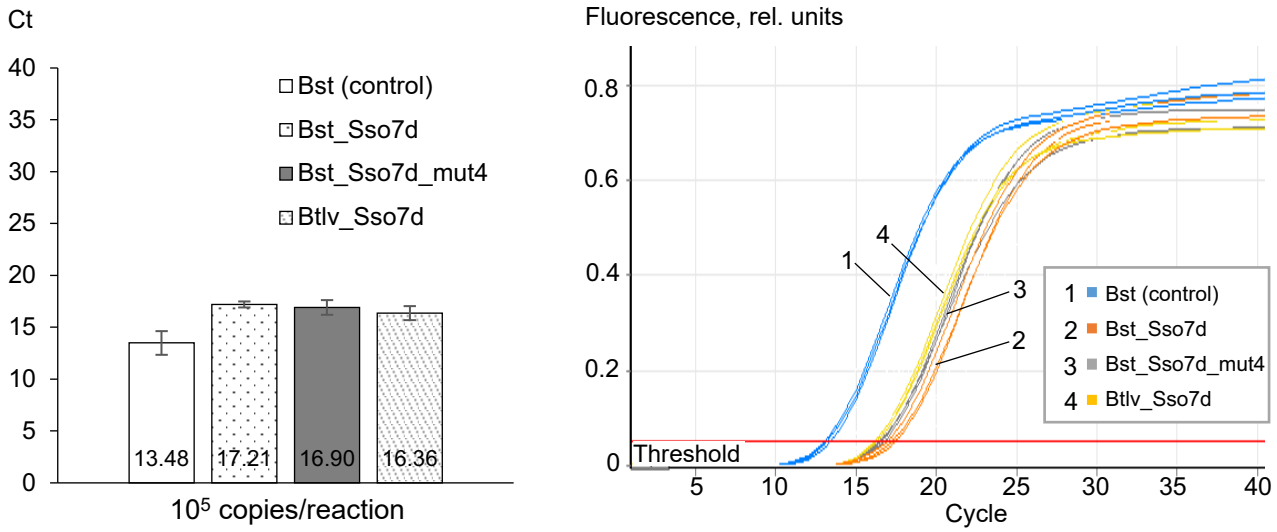

Fig. 3. Evaluation of the activity of the obtained polymerases in the AmpliSens *SARS-CoV-2*-IT reagent kit (mean threshold cycle values).

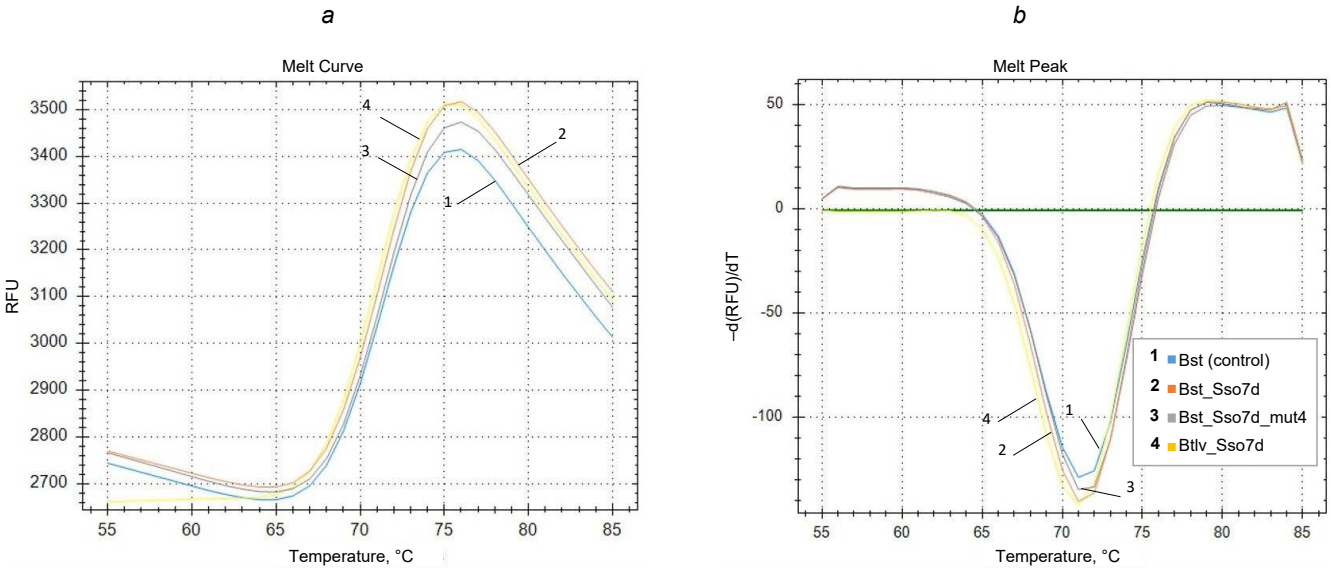

Fig. 4. Thermostability of chimeric polymerases.
a — thermal denaturation profiles; *b* — derivatives of fluorescence values with respect to temperature.

conducted only using the Btlv_Sso7d enzyme. The resistance of the Btlv_Sso7d polymerase to inhibitors from biological material (mucus from oropharyngeal swabs, salts from urine), components of the transport medium (transport medium for storing and transporting respiratory swabs (RU No. FSS 2009/05011)), and the lysis solution (1M guanidine hydrochloride) was also tested on 12 clinical urine samples and 12 oropharyngeal mucosa swab samples containing mumps virus RNA at a concentration of 10^5–10^8 copies/mL, isolated by three different methods: a precipitation-based method using the RIBO-prep kit; the EDEM express method; treatment with a 1M guanidine hydrochloride solution (for disinfecting the biological material) without subsequent washes. All isolated samples were then compared using the LAMP method without the addition of reverse transcriptase (MMLV) (**Fig. 6**).

When using Btlv_Sso7d polymerase, unlike the control enzyme, the LAMP reaction maintains its efficiency under all studied conditions, and all biological samples containing mumps virus RNA used in the study are identified as positive. There is also a less significant increase in Ct values when analyzing samples after nucleic acid extraction using the EDEM express method, compared to the control enzyme. This confirms that the Btlv_Sso7d polymerase possesses sufficient reverse transcriptase activity and increased inhibitor tolerance for the qualitative detection of mumps virus RNA (Fig. 6).

Due to its higher tolerance to inhibitors, the use of the new Btlv_Sso7d polymerase in isothermal amplification reactions based on the LAMP method, combined with rapid nucleic acid extraction methods that include only the lysis stage, will expedite molecular diagnostics and make it possible to use LAMP-based tests in field conditions or at the bedside of a patient.

Discussion

The LAMP method is a promising direction in the molecular diagnosis of infectious diseases. The main advantage of the method is the shorter analysis time: the amplification stage takes only 25–40 minutes, while the sensitivity and specificity of the method are comparable to that of PCR [1, 2]. The isothermal reaction mode allows for the use of simpler equipment for conducting the reaction: a thermostat with a fluorescence detection module instead of an amplifier.

Besides the actual amplification stage, any analysis most often includes the nucleic acid extraction stage. The extraction of nucleic acids is carried out to eliminate the main amplification inhibitors that may be present in biological samples. On average, depend-

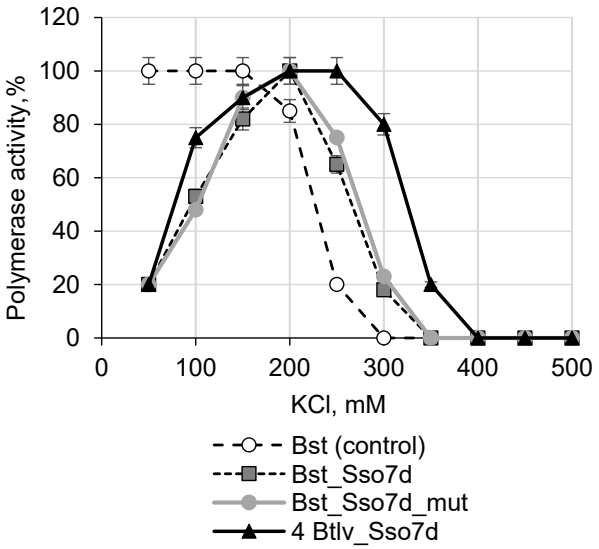

Fig. 5. Optimal KCl concentration for chimeric polymerases.

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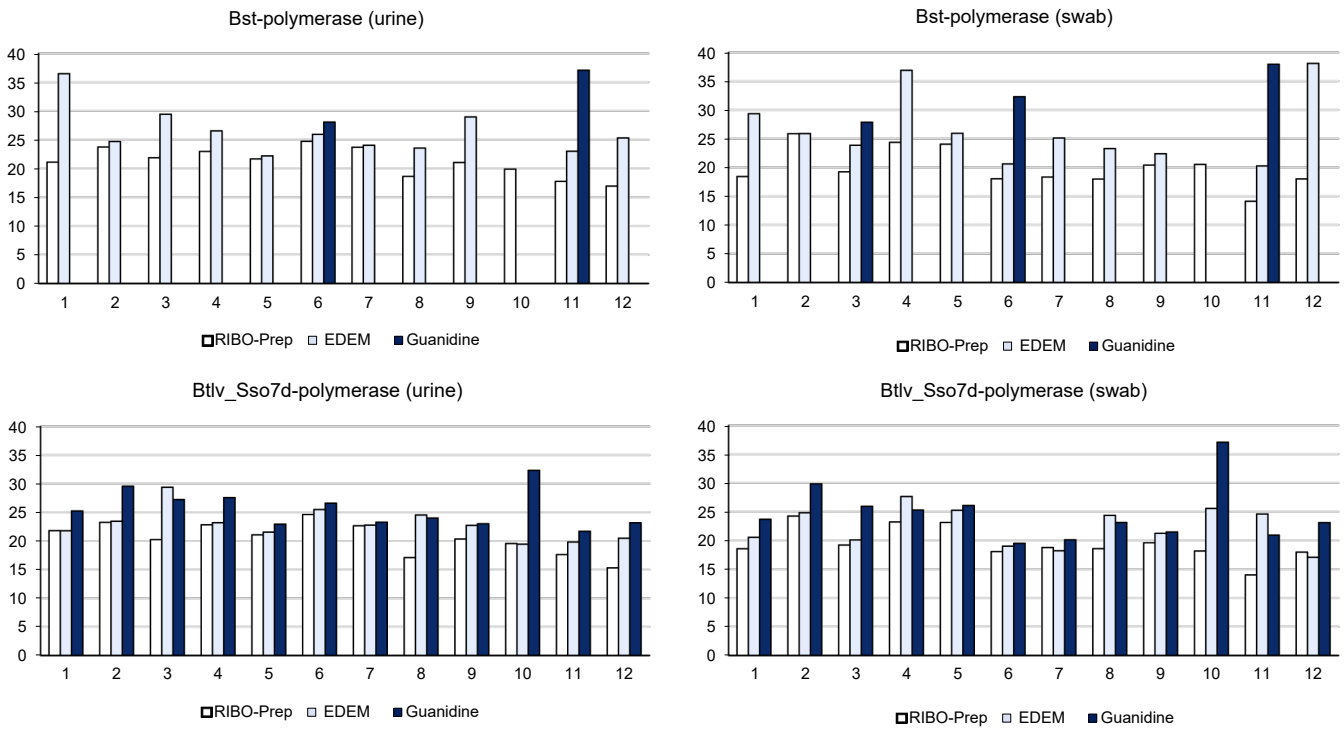

Fig. 6. Detection of the mumps virus in biological samples using LAMP with various polymerases without the addition of reverse transcriptase.

ing on the method used, the extraction stage can take from 1 to 3 hours. Reducing this stage by using express nucleic acid extraction methods (10–30 minutes) will significantly decrease the overall research time. For the successful implementation of such a strategy, the development and application of enzymes resistant to the main amplification inhibitors are necessary.

The Bst polymerases obtained in this study, containing the DNA-binding domain Sso7d: Bst_Sso7d and Bst_Sso7d_mut4, demonstrated reduced stability in the presence of whole blood and urine in the reaction mixture compared to the control enzyme without additional domains. At the same time, the Btlv_Sso7d enzyme is characterized by higher resistance to the presence of whole blood (10 vol. %), plasma (1 vol. %), and urine (10 vol. %). All enzymes retain their activity in the presence of mucin up to 0.5 mg/ml. These data demonstrate that the addition of the DNA-binding

Table 5. Comparison of the effects of inhibitors on the detection of mumps virus RNA using the tested enzymes

Inhibitors		Detection of mumps virus RNA			
Name	Concentration	Bst-polymerase (control)	Bst_Sso7d	Bst_Sso7d_mut4	Btlv_Sso7d
Whole blood, vol. %	0	+	+	+	+
	0,5	+	+	+	+
	1,0	+	+	+	+
	2,5	+	–	–	+
	5,0	+	–	–	+
	10,0	–	–	–	+
Blood plasma, vol. %	0	+	+	+	+
	1	–	–	–	+
Mucin, mg/ml	0	+	+	+	+
	0,2	+	+	+	+
	0,5	+	+	+	+
Urine, vol. %	0	+	+	+	+
	5	+	–	–	+
	10	–	–	–	+

domain did not lead to increased resistance to the inhibitory effect of urine and whole blood in the case of Bst polymerase. Similar studies have been published in the literature, indicating the opposite effect when using various Bst-like polymerases [17, 18, 30, 31]. However, the related chimeric enzyme Btlv_Sso7d obtained in this work demonstrates high resistance to the inhibitory effect of whole blood, plasma and urine components on the LAMP reaction. Such a difference in results may be explained by the characteristics of the genetic engineering constructs, the presence or absence of additional spacers between the DNA-binding domain and the polymerase, the structure of these spacers, and the conditions of cultivation and purification of recombinant proteins. The obtained data demonstrate the need for further detailed studies in this area to determine the influence of DNA-binding domains on the properties of chimeric enzymes in each specific case and to establish the relationships between the structure and functions of chimeric enzymes.

Conclusion

In a bacterial expression system based on *E. coli* cells, recombinant chimeric enzymes with displacing activity have been obtained: Bst_Sso7d, Bst_Sso7d_mut4 and Btlv_Sso7d. The developed protocols for obtaining and purifying the enzymes allow for the production of soluble enzymes with a yield of up to 25% of the total collected cell mass. In LAMP reactions, chimeric polymerases demonstrated similar activity to Bst polymerase without the Sso7d domain. At the same time, Btlv_Sso7d polymerase is characterized by increased reverse transcriptase activity and resistance to inhibitors, which allows it to be used in reagent kits for the diagnosis of infectious diseases by the LAMP method in combination with express nucleic acid extraction through thermal inactivation or lysis in the presence of guanidine chloride without subsequent washes from the components of the biological material and lysis solution. This significantly reduced the required time for the analysis.

As the demand for rapid and accurate diagnosis of infectious diseases grows, the use of LAMP methods, and consequently, Bst polymerase analogs with improved properties, will only increase.

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ORIGINAL RESEARCHES

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Characteristics of salmonellosis pathogens circulating in Primorsky Krai before and during the COVID-19 pandemic

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Abstract

Introduction. To date, among intestinal infections, salmonellosis is a pressing health problem worldwide, including in the Russian Federation, causing acute infectious outbreaks of disease.

Objective of the study: to assess the impact of the COVID-19 pandemic on the epidemiological patterns and molecular biological characteristics of salmonella infection in Primorsky Krai for 2019–2023.

Materials and methods. A retrospective epidemiological analysis of salmonellosis incidence during the COVID-19 pandemic in the population of Primorsky Krai was conducted. Plasmid characteristics of non-typhoidal *Salmonella* strains and their serotype were established using alkaline lysis and serological typing methods.

Results. During the COVID-19 pandemic, both in the Russian Federation and in Primorsky Krai, the registration of salmonellosis was significantly lower than in the pre-pandemic period, and the seasonality of salmonellosis shifted to the autumn months. An inverse relationship of medium strength was revealed between the monthly incidence of salmonellosis and COVID-19. No significant changes in the plasmid spectrum and in the proportion of registration by serogroups and serotypes of *Salmonella* were observed. It was shown that salmonellosis infection in Primorsky Krai is mainly caused by *S. enteritidis* strains with a plasmid characteristic of 38 MDa, 38:1.4 MDa, 38:2.3 MDa, 38:4.4 MDa, rare variants of plasmid types were also detected, the influence of the COVID-19 pandemic on the emergence of new plasmid types has not been established.

Conclusion. The presented results of the study on the molecular biological characteristics of *Salmonella* circulating in Primorsky Krai during the COVID-19 pandemic will open up prospects for understanding the ecological and epidemiological patterns of the development of the epidemic process of salmonella infection.

Keywords: *salmonellosis, Salmonella, serogroup, serotype, plasmid type, COVID-19 pandemic, SARS-CoV-2 virus*

Ethical approval. The study was conducted with the informed consent of patients. The research protocol was approved by the Ethics Committee of the Somov Research Institute of Epidemiology and Microbiology of Rospotrebnadzor (protocol No. 3 February 03.2022).

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Оригинальное исследование
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Характеристика возбудителей сальмонеллёзов, циркулирующих в Приморском крае до и во время пандемии COVID-19

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Аннотация

Введение. Сальмонеллёзы, для которых характерны острые вспышки заболеваемости, являются актуальной проблемой для здравоохранения.

Цель исследования: оценить влияние пандемии COVID-19 на эпидемиологические закономерности и молекулярно-биологические характеристики сальмонеллёзных инфекций в Приморском крае за 2019–2023 гг.

Материалы и методы. Проведён ретроспективный эпидемиологический анализ заболеваемости населения Приморского края сальмонеллёзами в период пандемии COVID-19. Методами щелочного лизиса и серологического типирования установлены плазмидная характеристика штаммов нетифоидных *Salmonella* и их серотип.

Результаты. Во время пандемии COVID-19 как в России, так и в Приморском крае регистрация сальмонеллёзов была значительно ниже, чем в допандемический период, а сезонность сальмонеллёзов сдвинулась на осенние месяцы. Выявлена обратная связь средней силы между данными помесячной заболеваемости сальмонеллёзами и COVID-19. Особых изменений в плазмидном спектре и в доле регистрации по серогруппам и серотипам *Salmonella* не наблюдалось. Показано, что сальмонеллёзные инфекции в Приморском крае в основном вызваны штаммами *S. enteritidis* с плазмидной характеристикой 38 МДа, 38:1,4 МДа, 38:2,3 МДа, 38:4,4 МДа, выявлялись также редкие варианты плазмидных типов. Влияние пандемии COVID-19 на появление новых плазмидных типов не установлено.

Заключение. Представленные результаты исследования об эпидемиологической и молекулярно-биологической характеристике *Salmonella*, циркулирующей в Приморском крае в период пандемии COVID-19, открывают перспективы для понимания эколого-эпидемиологических закономерностей развития эпидемического процесса сальмонеллёзов.

Ключевые слова: *сальмонеллёз, Salmonella, серогруппа, серотип, плазмидный тип, пандемия COVID-19, вирус SARS-CoV-2*

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Introduction

Salmonelloses are caused by various serotypes of bacteria of the genus *Salmonella*, are classified as polyetiological infectious diseases in humans and animals, and pose a significant public health problem worldwide, contributing to an increased economic burden. Depending on the health status of the host organism and the serotype of *Salmonella*, as well as the bacteria's resistance to antimicrobial agents, 11–20 million cases of salmonellosis are registered worldwide annually, resulting in up to 161,000 deaths [1].

Nowadays, salmonellosis is one of the most common bacterial intestinal infections, with a trend towards increasing outbreaks of the disease. According to official statistics, in recent years, the average incidence rate in Russia was 13.61 per 100,000 population in 2021[1], 17.1 in 2022, and 21.45 in 2023, with the rate in certain territorial districts exceeding the national average.

In the Primorsky Krai over the past decade, against the background of significant changes in the etiological structure of intestinal infections towards an increase in the share of rotavirus and norovirus infections and a decrease in the proportion of diseases such as viral hepatitis A and E and shigellosis, the percentage of salmonellosis has practically not changed and fluctuates within 10% [2].

In 2020, an emergency situation arose due to the development of the COVID-19 pandemic caused by the SARS-CoV-2 virus, which significantly affected the incidence of other infectious diseases with various transmission mechanisms [3–9]. The literature mainly presents studies showing the impact of COVID-19 on acute respiratory viral infections. Only a few publications address the impact of the COVID-19 pandemic on the epidemiological patterns and microbiological characteristics of infections related to the intestinal group [4], while there is virtually no data on its influence on the epidemiological patterns of salmonellosis development in Russia.

The aim of the study is to assess the impact of the COVID-19 pandemic on the epidemiological patterns and molecular-biological characteristics of non-typhoidal salmonellosis in the Primorsky Krai from 2019 to 2023.

Materials and methods

A retrospective epidemiological analysis of the incidence of salmonellosis and COVID-19 among the population of Primorsky Krai was conducted based on data from the federal state statistical observation for Primorsky Krai — "Information on Infectious and Parasitic Diseases" (Form No. 2) for the years 2009–2023.

The spectrum of plasmid types in salmonella strains was determined by the alkaline lysis method [10]. Known plasmid types RP4 (38 MDa), pBR322 (2.9 MDa), pVM82 (82 MDa) were used as molecular weight standards.

When performing the serological typing method, diagnostic salmonellosis adsorbed dry sera for agglutination reaction (Petsal) were used in accordance with the Kaufmann–White antigenic scheme [11].

Statistical data processing was carried out using the Microsoft Excel 2010 and Statistica v. 10 programs. The critical significance level p for testing statistical hypotheses was set at 0.05.

Results

The period from 2009 to 2012 in the Primorsky Krai was characterized by an increase in the incidence

[1] On the state of sanitary and epidemiological welfare of the population in the Russian Federation in 2023: State Report. Moscow; 2024. 368 p.

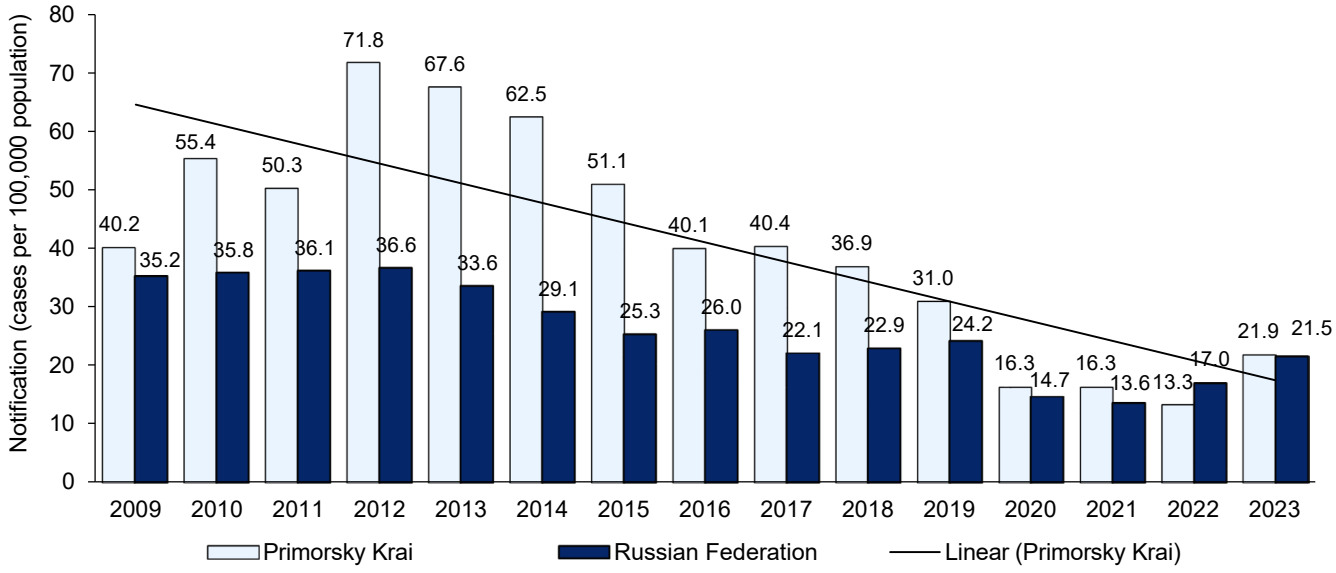

Fig. 1. The dynamics of salmonellosis incidence in Primorsky Krai and the Russian Federation in 2009–2023.

of salmonellosis, with a peak registered in 2012 at 71.8 per 100,000 population. In the following years, a trend towards a decrease in morbidity was observed, and by 2022, its level had dropped to 13.3 per 100,000 population (the growth rate was –4.2), whereas in Russia, the incidence of salmonellosis was more stable (the growth rate was –3.4). At the same time, the incidence rates in Primorsky Krai exceeded the national data from 2009 to 2019, and starting from 2020, they almost leveled off (**Fig. 1**). In 2023, an increase in morbidity was observed both in Primorsky Krai and in Russia as a whole (up to 21.6 per 100,000 population).

Before and during the COVID-19 pandemic, the lowest incidence of salmonellosis infections in the Primorsky Krai region was recorded in the municipal districts of Terneisky, Kavalerovsky, Lazovsky, Olginsky, Partizansky, and the city of Fokino (**Fig. 2**). It should be noted that during the peak of the pandemic, *S. enterica* strains were not isolated in these areas.

A high level of salmonellosis, both before and during the COVID-19 pandemic, was recorded in the cities of Vladivostok, Ussuriysk, Spassk-Dalny, and in the Khorolsky and Khasansky districts. The period of maximum disease incidence occurred in 2019, and in 2023, a gradual recovery to the usual level was recorded.

In the monthly dynamics (**Fig. 3**), the incidence of salmonellosis before and during the COVID-19 pandemic from 2018 to 2019 was predominantly recorded in the summer-autumn period (June–October). At the

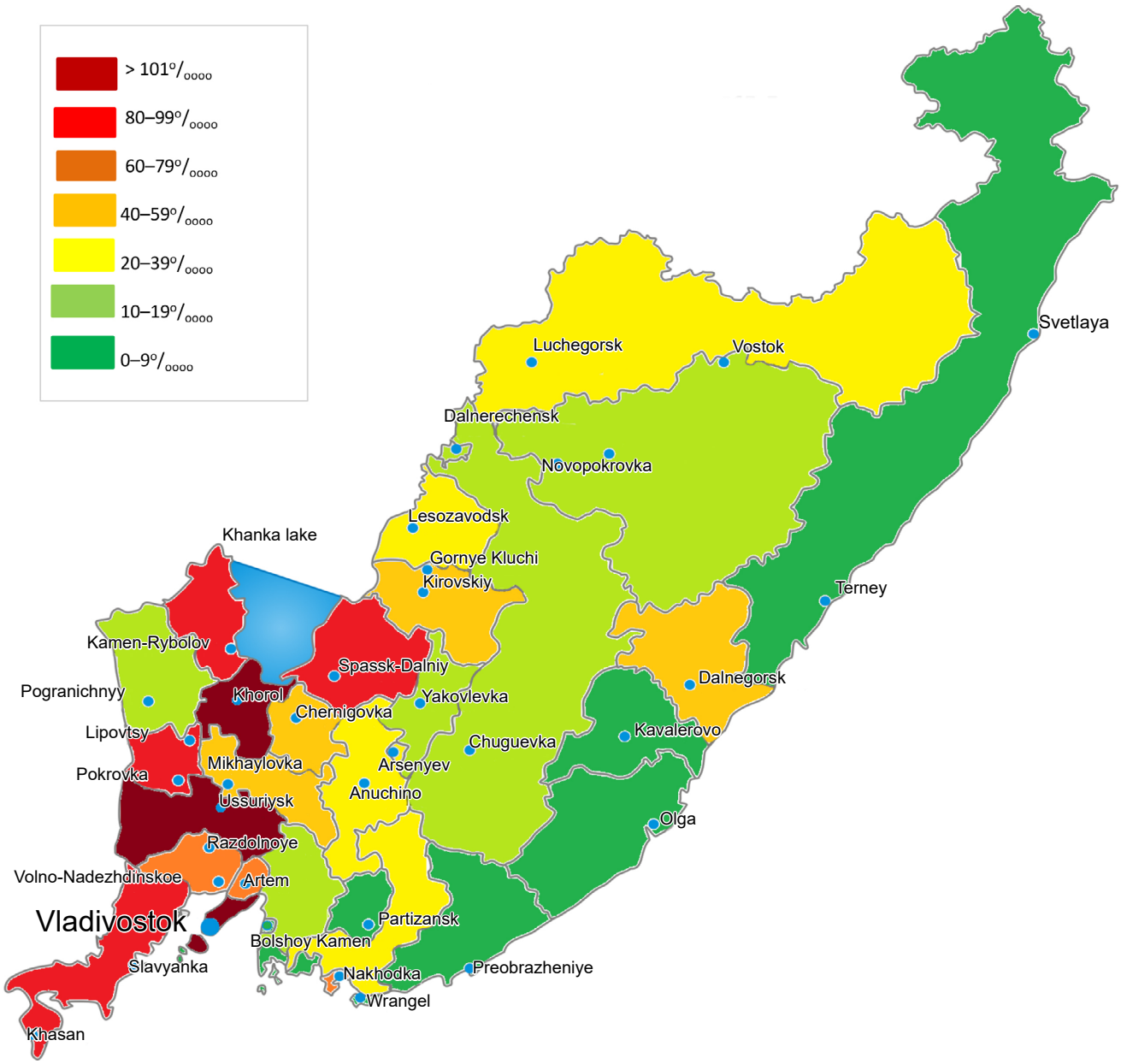

Fig. 2. Incidence of salmonellosis in the administrative territories of Primorsky Krai in 2019–2023 (per 100,000 population).

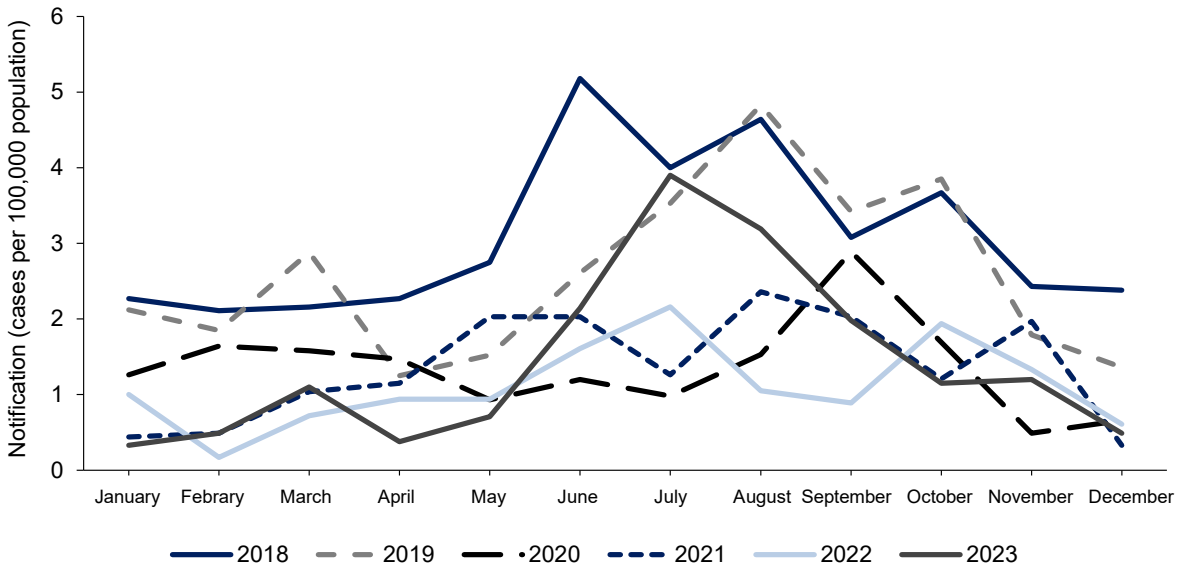

Fig. 3. Monthly dynamics of salmonellosis incidence in Primorsky Krai from 2018 to 2023.

height of the COVID-19 pandemic, starting in 2020, the seasonality shifted to the autumn months (September–October). In 2021, the highest peaks of salmonellosis incidence occurred in May and June, August and September, as well as November. In 2023, the incidence of salmonellosis in the Primorsky Krai region began to recover, and its seasonality was observed in the summer-autumn period (June–October). Similar results are reported by other authors as well [4].

Interesting data were obtained from the joint analysis of monthly COVID-19 morbidity in the same years. Thus, in the dynamics of COVID-19 and salmonellosis incidence for the years 2021–2022 (**Fig. 4**), four periods can be identified in the change of incidence levels. From January to October 2021, there was a decline in COVID-19 cases, while the incidence of salmonellosis increased. From November 2021 to March 2022, the incidence of COVID-19 increased, while the incidence of salmonellosis decreased from December 2021 to February 2022 From March to July 2022, there was a significant increase in the incidence of salmonellosis. From August to September 2022, there was an increase in COVID-19 cases alongside a decrease in reported salmonellosis cases. Correlation analysis of the presented data using Spearman's method revealed a moderate inverse relationship between the monthly incidence data for both infections ($r = 0.5$; $p < 0.05$).

It has been established that in the Primorsky Krai, as well as in most regions of the Russian Federation and worldwide, Salmonella of serogroup D with the dominant *S. enteritidis* serotype holds the leading position, followed by serogroups C and B with insignificant share values (serogroup B — 9.1 ± 1.3%; serogroup C — 9.7 ± 1.3%). The last place is occupied by serogroup E, which was not registered in 2022. It should be noted that the lowest incidence of disease caused by other serogroups of *S. enterica* was recorded in 2022 (**Fig. 5**).

Nowadays, the main serotypes identified in salmonellosis are *S. enteritidis*, *S. typhimurium* and *S. infantis*. Since 1995 and up to the present, despite the diversity of plasmid types in the leading serotype *S. enteritidis*, the three main plasmid types — 38, 38:1.4, and 38:4.4 MDa—continue to hold prevalent significance (**Table**). Other, less frequently isolated plasmid types (38:2.3, 38:2.3:1.4, 38:2.6:1.4, 38:2.6:14, 38:3.0:1.4, 38:30, 38:30:1.4, 38:30:2.3 MDa — epidemic pool) have also retained their significance in shaping the epidemic situation. The results of the study showed that the number of salmonellosis cases caused by *S. enteritidis* with

Dynamics of the number of plasmid types of S. enteritidis isolated in the Primorsky Krai from 2009 to 2023

Plasmid types (MDa)	Year															Total strains	Isolate source
	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023		
38	220	128	110	397	313	234	211	140	93	76	96	41	31	37	26	2153	v, fp, env.
38:1.4	297	323	299	343	538	349	309	154	186	138	66	17	17	12	17	3065	v, fp, env.
38:2.3	7	5	9	12	8	2	5	7	13	10	11	9	3	2	–	103	v, fp, env.
38:4.4	82	368	267	179	111	132	107	137	119	82	50	13	24	1	8	1760	v, fp

Note. v — patients; fp — food products; env. — environment.

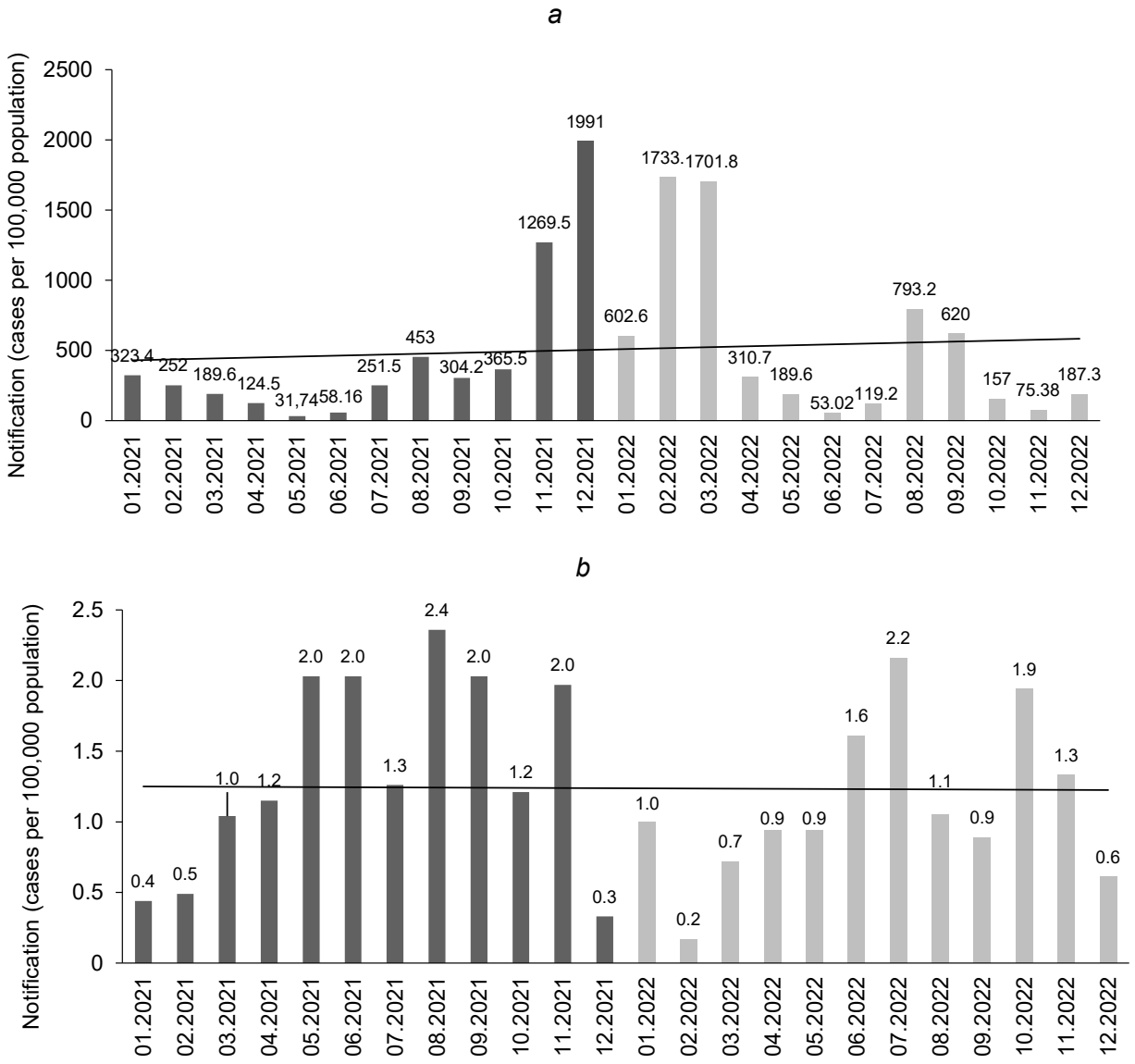

Fig. 4. Comparative analysis of the monthly dynamics of COVID-19 (*a*) and salmonellosis (*b*) incidence from 2021 to 2022.

plasmid types 38, 38:1.4, 38:2.3, and 38:4.4 MDa gradually decreased until 2019, and since the beginning of the COVID-19 pandemic, it has been reduced by half (**Table 1**).

It is important to note that both before and during the COVID-19 pandemic, rare plasmid type variants of the *S. enteritidis* serovar were identified: 38:26:2.6, 3.2:2.0, 38:3.8:3.0, 5.6:3.8:3.2:2.0, 38:4.4:3.2 50:38:20, 38:5.0:3.8:3.0, and 50:8.0:4.0:3.5:1.4 MDa. The impact of the epidemiological situation during the COVID-19 pandemic on the emergence of new plasmid types has not been established by us.

The second most significant serotype in the etiology of salmonellosis in the Primorsky Krai is *S. typhimurium*. From 2019 to 2023, more than 90 strains of *S. typhimurium* isolated from various sources were studied. All infections were of a foodborne nature, and no hospital strains were isolated. The most frequently registered strains were those with a single plasmid type — 60 MDa (14.3%), followed by the plasmid type 4.0:3.2 MDa (13.2%), which first appeared in 2015 (isolated from a patient in the city of Ussuriysk).

In the *S. infantis* serotype circulating in the Primorsky Krai region, the 100 MDa plasmid type predominated (72%), isolated from food products. Strains with other plasmid types were rarely isolated.

Discussion

Intestinal infections are a pressing issue worldwide due to the extremely widespread nature of this pathology, high morbidity rates, and, consequently, significant economic damage [12].

Salmonella infections and COVID-19 have different modes of transmission, but in some cases, they exhibit comparable symptoms [13, 14]. Moreover, the immunological response triggered by the SARS-CoV-2

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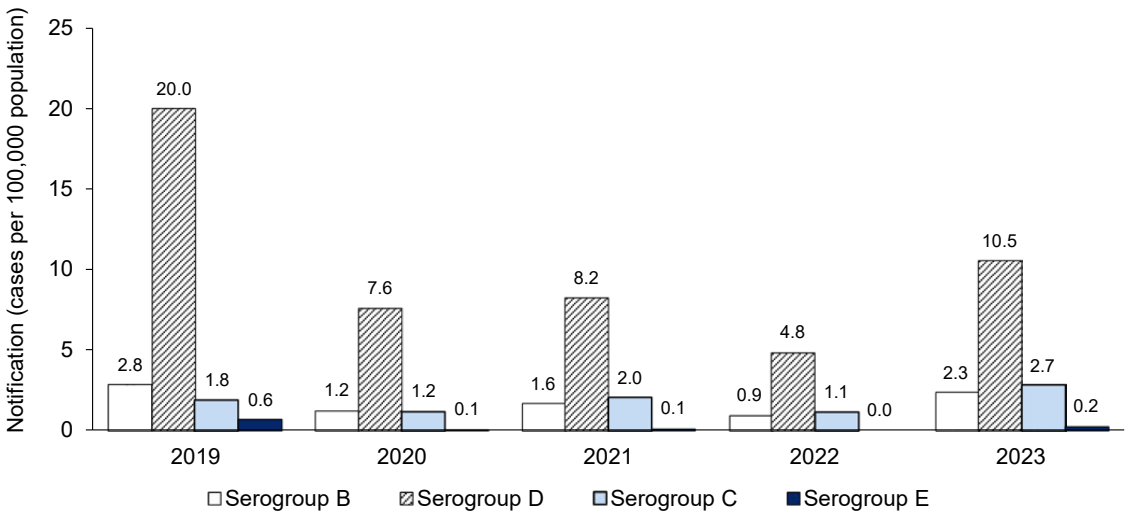

Fig. 5. Dynamics of the incidence of the population with individual serogroups of *Salmonella enterica* in Primorsky Krai from 2019 to 2023.

virus can influence the severity of bacterial infections, including salmonellosis [15]. It was later established that these factors have serious implications related to the onset and treatment of salmonellosis [16–19]. Cases of co-infection with Salmonella and COVID-19 have been reported in 9 studies worldwide [20], some of which were found in patients from Pakistan [21], Turkey [22] and Japan [23]. Patients with coinfection face more severe consequences and increase the complexity of therapeutic decisions necessary to address this issue.

In 2020, the development of the COVID-19 pandemic worldwide significantly affected the incidence rates of other infections with different transmission mechanisms. Thus, when comparing with the average annual indicator from 2016 to 2023, the incidence of infections with an aerosol transmission mechanism from 2020 to 2023 in Primorsky Krai was higher than the long-term average, while the incidence of intestinal infections was significantly lower. The lowest rates were recorded in 2020–2021, however, in the following years, there is a trend towards an increase.

The incidence rates of vector-borne infections for 2020–2023 were also significantly lower than the multiyear average. Similar data were obtained for the incidence of infections transmitted due to person-to-person contact.

It has been established that in all groups, except for air-borne infections, the incidence during the COVID-19 pandemic years, compared to the average indicators of previous years, has significantly decreased.

Salmonelloses in the Primorsky Krai continue to play an important role in shaping infectious morbidity, as evidenced by the increase in outbreaks and the exceedance of the average morbidity rate compared to Russia as a whole. It should be noted that during the peak of the COVID-19 pandemic, there was a decrease in the growth of salmonellosis cases. The lowest incidence was registered in the Terneysky, Kavalerovsky, Lazovsky, Olginsky, Partizansky districts and Fokino, however, in the cities of Vladivostok, Ussuriysk, Spassk-Dalny, Khorolsky, and Khasansky districts, a high level of salmonellosis was noted. During the pandemic, a divergent trend was observed in the monthly dynamics of COVID-19 and salmonellosis incidence compared to the pre-pandemic period. The decrease in the registration of salmonellosis cases may be related to the isolation of the population during the pandemic, the transition to remote work formats, the quarantine of multidisciplinary medical organizations due to the detection of coronavirus infection cases, and the re-profiling of clinical hospitals for the diagnosis and specialized treatment of patients with confirmed diagnoses. Nevertheless, the influence of the biogeocenosis on the self-regulation of various microorganisms as a result of integrative-competitive relationships between them, which is reflected in the changing trends in the dynamics of morbidity from specific infections and the structure of infectious pathology, cannot be excluded in the formation of the existing epidemiological situation [24, 25].

Despite the diversity of plasmid types in the leading serotype *S. enteritidis*, three main plasmid types — 38, 38:1.4, and 38:4.4 MDa — play a dominant role in the development of salmonellosis, as confirmed by the research results. It has been established that salmonellosis infections in the Primorsky Krai, both in the pre-pandemic period and at the height of the COVID-19 pandemic, were mainly caused by *S. enteritidis* strains with plasmid characteristics of 38, 38:1.4, 38:2.3, and 38:4.4 MDa. Much like in the pre-pandemic period, rare variants of plasmid types were also identified. The impact of the epidemic situation during the COVID-19 pandemic on the emergence of new plasmid types has not been established.

Conclusion

The obtained data on the epidemiological and molecular-biological characteristics of the pathogens of salmonellosis circulating in the Primorsky Krai during the pre-pandemic period and at the height of the COVID-19 pandemic open up prospects for uncovering the ecological and epidemiological patterns of the epidemic process, which will allow for making informed managerial decisions for implementing control over salmonellosis infection.

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Results of reconnaissance epizootiological monitoring for West Nile fever in certain regions of European Russia and the Urals in 2024

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Abstract

Introduction. Climate warming contributes to the intensification of epizootic and epidemic processes of West Nile fever (WNF). In southern Russia, the activity of the epizootic process is recorded annually, but in the central region of the European part of the country and in the Urals, the enzootic circulation of the West Nile virus (WNV) has not been confirmed in the territory of 20 subjects.

The aim of the study is to investigate zoological and entomological material for WNV infection to confirm the ongoing epizootic process in old WNV foci and in previously non-endemic areas.

Materials and methods. Field samples were collected in 2024 in 19 subjects in accordance with the methods regulated in normative documents. The material was studied using the reverse transcription polymerase chain reaction method.

Results. In total, during the 2024 field season, 5,419 samples of field samples were examined: 684 samples of birds from 74 species, 455 samples of small mammals from 13 species, 45 samples of frogs from 1 species, 3,665 samples of blood-sucking mosquitoes from 33 species (93,438 specimens), and 570 samples of ixodid ticks from 17 species (4,809 specimens). Markers of WNV in field samples were detected in 7 subjects from 3 federal districts. In the Kirov and Chelyabinsk regions and the Republic of Mordovia, evidence of the ongoing epizootic process of WNF has been obtained for the first time. WNV RNA was detected in 6 (0.5%) out of 1184 tested samples of vertebrate animals and in 27 (0.6%) out of 4235 samples of arthropods. The level of individual infection was 0.03% in blood-sucking mosquitoes, 0.06% in ixodid ticks, and 0.9% in birds.

Conclusion. The results of the studies confirm the enzootic circulation of WNV in the territories of the Southern, Volga and Ural Federal Districts.

Keywords: *West Nile fever, West Nile virus, epizootic process, infection, natural reservoir, vectors*

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 study was approved by the Bioethics Committee of the Volgograd Plague Control Research Institute (Protocol No. 1 dated January 14, 2024).

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ORIGINAL RESEARCHES

Оригинальное исследование
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Итоги рекогносцировочного эпизоотологического мониторинга лихорадки Западного Нила на отдельных территориях европейской части России и Урала в 2024 году

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Аннотация

Актуальность. Потепление климата способствует интенсификации эпизоотического и эпидемического процессов лихорадки Западного Нила (ЛЗН). На юге России активность эпизоотического процесса регистрируют ежегодно, но в центральном регионе европейской части страны и на Урале энзоотическая циркуляция вируса Западного Нила (ВЗН) не подтверждена на территории 20 субъектов.

Цель работы — исследовать зоолого-энтомологический материал на инфицированность ВЗН для подтверждения течения эпизоотического процесса в «старых» очагах ЛЗН и на ранее неэндемичных территориях.

Материалы и методы. Полевой материал собирали в 2024 г. в 19 субъектах в соответствии с регламентированными в нормативных документах методами. Исследование материала проводили методом полимеразной цепной реакции с обратной транскрипцией.

Результаты. Всего в полевой сезон 2024 г. исследовано 5419 проб полевого материала: 684 пробы птиц 74 видов, 455 проб мелких млекопитающих 13 видов, 45 проб лягушек 1 вида, 3665 проб кровососущих комаров 33 видов (93 438 экземпляров), 570 проб иксодовых клещей 17 видов (4809 экземпляров). Маркеры ВЗН в полевом материале обнаружены в 7 субъектах из 3 федеральных округов. В Кировской, Челябинской областях и Республике Мордовия доказательства течения эпизоотического процесса ЛЗН получены впервые. РНК ВЗН выявлена в 6 (0,5%) из 1184 исследованных проб позвоночных животных и в 27 (0,6%) из 4235 проб членистоногих. Уровень индивидуальной заражённости кровососущих комаров составил 0,03%, иксодовых клещей — 0,06%, птиц — 0,9%.

Выводы. Результаты исследований подтверждают энзоотичную циркуляцию ВЗН на территории Южного, Приволжского и Уральского федеральных округов.

Ключевые слова: *лихорадка Западного Нила, вирус Западного Нила, эпизоотический процесс, инфицированность, природный резервуар, переносчики*

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). Исследование одобрено комитетом по биоэтике Волгоградского научно-исследовательского противочумного института Роспотребнадзора (протокол № 1 от 14.01.2024).

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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

West Nile fever (WNF) is an enzootic, natural focal, vector-borne infectious disease caused by the West Nile virus (WNV) from the genus *Orthoflavivirus*. Certain bird species are the reservoir host of the pathogen, while the vectors are blood-sucking mosquitoes [1].

Climate warming over the past decades has contributed to the transformation of many ecosystems on the planet, leading to changes in the natural habitats of various animal species, including reservoirs and vectors of zoonotic infections. The increase in temperature leads to an increase in the replication rate of the pathogen, an extension of the stay of migratory birds in nesting areas, as well as accelerated development, extended activity periods, and the expansion of the ranges of blood-sucking mosquito vectors [2, 3]. All of the mentioned contributes to the intensification of epizootic and epidemic processes of WNF in natural focal areas and the spread of WNV.

Targeted monitoring for the infection rates in reservoir and vector populations makes it possible to identify signs of the activation of the epizootic process, promptly carry out measures to reduce vector populations, and inform the public about the necessity to use individual and collective protection measures against mosquito bites. Moreover, effective monitoring allows for dynamic observations of the activity of natural foci of zoonotic infections.

At the beginning of the study of WNF, it was commonly accepted that the range of WNV was limited to the territories of the equatorial, subequatorial, tropical, subtropical and southern parts of the temperate climate zones. On the territory of the former USSR, it covered the southern part of the European part of Russia, Belarus, Moldova, Ukraine, Azerbaijan, Georgia, Tajikistan, Kyrgyzstan, Kazakhstan and Turkmenistan. For the first time in the USSR, the virus was isolated in 1963 from *Hyalomma plumbeum* ixodid ticks (now *Hyalomma marginatum*) in the Astrakhan region, as well as from a sandpiper and a blackbird from Azerbaijan [4]. In the 1980s, the pathogen was detected in regions located significantly further north: in rooks and nidicolous birds from their nests in the Omsk region, in nidicolous birds from the Novosibirsk region, and in *Aedes vexans* mosquitoes from the Republic of Tatarstan. During the WNF outbreak in Moscow in 2021, the WNV RNA was detected in 14.2% of samples from the total number of examined blood-sucking mosquitoes, 68.0% of dead birds, and 32.0% of live birds [7]. The obtained data indicated a broader territorial spread of the WNF pathogen than was previously accepted [8]. As of early 2024, markers of WNF have been identified in samples from carriers and vectors in 52 entities across the territory of the Russian Federation. In southern Russia, the activity of the epizootic process is recorded annually. At the same time, the absence of positive findings in certain regions of the central part of European Russia and in the Urals draws attention — endemic circulation of WNV has not been confirmed in 10 entities of the Central Federal District (CFD), 6 in the Volga Federal District (VFD) and 4 in the Ural Federal District (UFD). In certain non-endemic territories, local cases of human WNF have been identified, indicating the presence of foci of this arboviral infection. Therefore, conducting active reconnaissance surveys aimed at clarifying the nosoareal is relevant.

The aim of this study is to investigate the zoological-entomological material for the presence of WNF to confirm the ongoing epizootic process in old foci of WNF and in previously non-endemic areas.

Materials and methods

The collection of field samples for research at the Reference Center for monitoring the WNF pathogen in the 2024 season was carried out from April to November in 19 entities of the Russian Federation (**Figure**) by employees of the Volgograd Research Anti-Plague Institute of Rospotrebnadzor, as well as by anti-plague institutions and the Centers for Hygiene and Epidemiology in the entities of the Russian Federation.

The capture of small mammals was carried out with the help of snap traps. Birds were hunted by employees of hunting farms through shooting, and the collection of fallen individuals was carried out by staff of zoological groups and researchers. Sampling of blood-sucking mosquitoes in open biotopes in household plots, along water bodies, in cemeteries, and in forests was carried out using automatic traps such as BG-sentinel-2 (Biogents AG), LovKom (ProTechnoSystems), Mosquito Magnet Executive (Woodstream), Black Kill M3000 (Black Kill), and an entomological net. Mosquitoes were captured in enclosed biotopes (chicken coops, pigsties, basements of multi-story buildings) with the help of battery-operated vacuum cleaners (BLV 18-200, Karcher and Tefal X-PERT 3.60 Versatile Handstick TY6975WO, Tefal) and exhausters. The collection of ixodid ticks was carried out using classical methods: in nature — with an entomological flag on vegetation, in populated areas — from animals (small and large livestock, dogs, cats).

The collected arthropods were delivered to the laboratory in thermal containers with cold packs, identified using the Stemi 2000C (Karl Zeiss) and MSP-1 (LOMO) stereomicroscopes on a cooled surface to the species level according to standard keys [9–12]. They were placed in 2 mL cryotubes.

Field samples were transported on dry ice or in car refrigerators at –20°C. For the analysis of climatic factors, data from the Federal Service for Hydrometeo-

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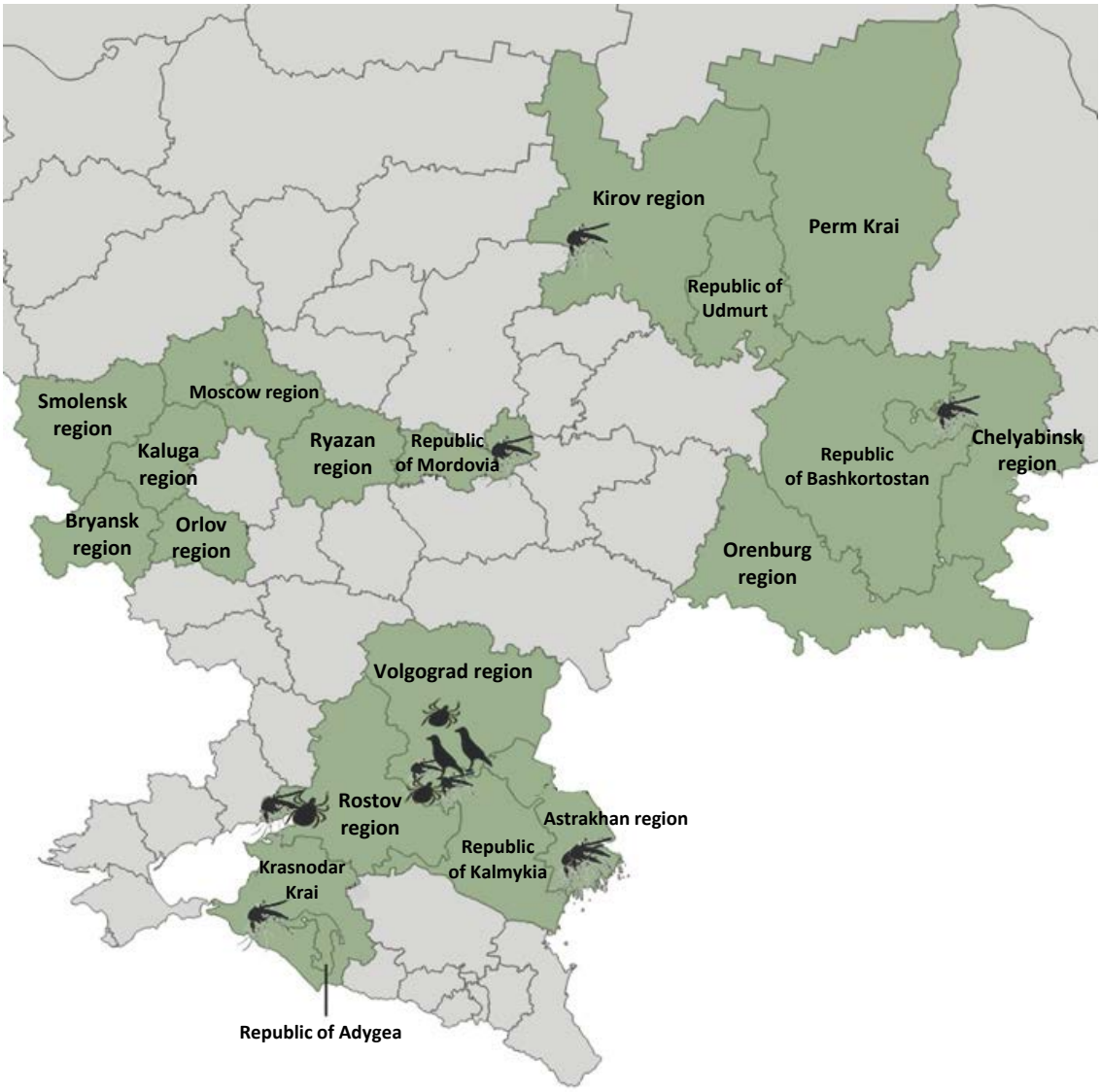

Entities where zoological and entomological material sampling was conducted in 2024, and points of positive WNV RNA findings from birds and arthropods.

rology and Environmental Monitoring of the Russian Federation were used[1].

Field samples were examined using the reverse transcription polymerase chain reaction method at the stationary laboratory of the Volgograd Research Anti-Plague Institute of Rospotrebnadzor. For the extraction of WNV RNA, suspensions of blood-sucking arthropods, as well as organs from birds, small mammals, and frogs (in a pooled sample from each individual – brain, kidneys, spleen) were prepared. The detection of WNV RNA was carried out using the AmpliSens WNV-FL reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor) according to the manufacturer's instructions. The determination of the WNV lineage in positive samples was carried out using the Ampligen-WNV-genotype-1/2/4 reagent kit (Volgograd Research Anti-Plague Institute of Rospotrebnadzor).

The infection rate of vertebrates was determined by calculating the proportion of positive samples from the total number of samples examined (%), and the individual infection rate of arthropods was calculated using the formula by V.N. Beklemishev [13]. Statistical processing of the materials and calculations were carried out using the Microsoft Excel program.

Results

A total of 5,419 field samples were examined during the 2024 field season: 684 bird samples from 74 species, 455 small mammal samples from 13 species, 45 frog samples from 1 species, 3,665 blood-sucking

[1] Newsletter of the Federal Service for Hydrometeorology and Environmental Monitoring: overview of the state and trends of climate change in Russia 2024 (December 2023 — November 2024). URL: http://downloads.igce.ru/climate_change_2/monitoring-klimata/Russia/2024/2024.pdf (In Russ.)

Table 1. Amount of field samples collected in 2024

Entity	Birds (specimens)	Small mammals (specimens)	Mosquitos		Ixodid ticks	
			specimens	samples	specimens	samples
Astrakhan region	–	–	7.231	254	559	81
Volgograd region	332	–	11.355	423	350	75
Rostov region	–	–	3.189	124	5	2
Republic of Kalmykia	–	–	598	25	–	
Republic of Adygea	7	9	2.926	112	370	67
Krasnodar Krai	1	–	9.736	341	–	–
Chelyabinsk region	–	–	2.430	97	–	–
Orenburg region	20	35	8.855	312	230	23
Republic of Bashkortostan	5	30	4.102	158	400	40
Perm Krai	24	54	3.034	142	779	70
Kirov region	–	75	7.067	276	200	21
Republic of Mordovia	44	60	5.252	254	109	24
Republic of Udmurt	23	73	2.995	120	222	20
Bryansk region	166	–	2.493	97	–	–
Smolensk region	4	35	5.650	214	400	20
Orlov region	20	15	2.736	117	310	33
Moscow region	17	18	4.054	168	57	8
Kaluga region	21	16	3.941	180	403	43
Ryazan region	0	35	5.794	251	415	43
Total	684	455	93.438	3665	4809	570

mosquito samples from 33 species (93,438 specimens), and 570 ixodid tick samples from 17 species (4,809 specimens). **Table 1** presents the amount of birds, small mammals, mosquitoes and ixodid ticks in each entity. In the Volgograd region, 45 frogs were also collected for the study.

The species composition, amount of samples and results of the studies are presented in **Table 2** and **Table 3**.

Markers of the WNF pathogen were found in field samples in 7 entities from 3 federal districts (figure), including in 3 entities (Kirov, Chelyabinsk regions and the Republic of Mordovia), where evidence of the epizootic process of WNF was obtained for the first time. In the specified territories, local cases of WNF were registered in the Chelyabinsk region in 2010 and 2011 [14], however, from 2010 to 2023, WNV markers were not detected in field samples.

WNV RNA was detected in 6 (0.5%) out of 1184 tested samples of vertebrate animals and in 27 (0.6%) out of 4235 samples of arthropods. In 1 sample, the RNA was typed as lineage 1 (Rostov region), in 25 samples as lineage 2 (Rostov, Volgograd, Kirov, Chelyabinsk, Astrakhan regions, Republic of Mordovia, Krasnodar Krai), and in 6 samples as lineage 4 (Volgograd region).

The overall level of individual infection rate (infection of each individual) among blood-sucking mosquitoes was 0.03%, among ixodid ticks it was 0.06%, and among birds it was 0.9%. When examining samples from small mammals and frogs, no markers of WNV were found.

From the CFD with negative results, 228 birds, 119 small mammals, 1,027 mosquito samples and 147 ixodid tick samples were examined.

The largest number of samples from the total number examined came from the Southern Federal District (SFD) and the VFD. From the SFD, 340 birds, 9 small mammals, 45 frogs, 1279 mosquito samples, and 225 ixodid tick samples were tested. WNV RNA was detected in 20 samples of mosquitoes (individual infection rate was 0.06%), 3 samples of ixodid ticks (0.24%), and 6 samples of birds (1.76%). Vertebrates from the VFD in the studies were represented by 116 birds and 327 small mammals. No positive findings from vertebrates were detected. Out of 1,262 mosquito samples collected in this district, WNV RNA was detected in 3 (individual infection rate — 0.01%). The results of the studies on 198 samples of ixodid ticks are negative.

97 samples of mosquitoes were delivered from the UFD for research. RNA markers of WNV were detected in 1 sample. The level of individual mosquito infection rate was 0.04%.

Discussion

One of the leading factors contributing to the activation of the epizootic process of WNF is the high

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Table 2. Results of WNV RNA testing in vertebrate species in 2024

Species	Amount of studied specimens	Amount of positive specimens
Birds		
White stork — *Ciconia ciconia* Linnaeus, 1758	1	0
Gray partridge — *Perdix perdix* Linnaeus, 1758	4	0
Common pheasant — *Phasianus colchicus* Linnaeus, 1758	5	0
Black grouse — *Lyrurus tetrix* Linnaeus, 1758	1	0
Rock dove — *Columba livia* Gmelin, 1789	84	0
Wood pigeon — *Columba palumbus* Linnaeus, 1758	2	0
Mottled duck — *Anas fulvigula* Ridgway, 1874	2	0
Gadwall — *Mareca strepera* Linnaeus, 1758	4	0
Tufted duck — *Aythya fuligula* Linnaeus, 1758	1	0
Greater white-fronted goose — *Anser albifrons* Scopoli, 1769	12	0
Greylag goose — *Anser anser* Linnaeus, 1758	6	0
Garganey — *Spatula querquedula* Linnaeus, 1758	7	0
Eurasian teal — *Anas crecca* Linnaeus, 1758	12	0
Mallard — *Anas platyrhynchos* Linnaeus, 1758	157	0
Common pochard — *Aythya ferina* Linnaeus, 1758	3	0
Northern Shoveler — *Spatula clypeata* Linnaeus, 1758	2	0
Eurasian Wigeon — *Mareca penelope* Linnaeus, 1758	2	0
Common merganser — *Mergus merganser* Linnaeus, 1758	2	0
Ruddy shelduck — *Tadorna ferruginea* Pallas, 1764	1	0
Common goldeneye — *Bucephala clangula* Linnaeus, 1758	3	0
Eurasian woodcock — *Scolopax rusticola* Linnaeus, 1758	54	0
Common snipe — *Gallinago gallinago* Linnaeus, 1758	1	0
Black-headed gull — *Chroicocephalus ridibundus* Linnaeus, 1766	9	0
Common tern — *Sterna hirundo* Linnaeus, 1758	1	0
Little tern — *Sternula albifrons* Pallas, 1764	2	0
Caspian gull — *Larus cachinnans* Pallas, 1811	1	0
Northern Lapwing — *Vanellus vanellus* Linnaeus, 1758	1	0
Eurasian dotterel — *Eudromias morinellus* Linnaeus, 1758	1	0
Common sandpiper — *Actitis hypoleucos* Linnaeus, 1758	2	0
Corncrake — *Crex crex* Linnaeus, 1758	1	0
Eurasian coot — *Fulica atra* Linnaeus, 1758	17	0
Gray heron — *Ardea cinerea* Linnaeus, 1758	12	2
Black-crowned Night heron — *Nycticorax nycticorax* Linnaeus, 1758	2	0
Purple heron — *Ardea purpurea* Linnaeus, 1766	4	0
Glossy ibis — *Plegadis falcinellus* Linnaeus, 1766	1	0
Great cormorant — *Phalacrocorax carbo* Linnaeus, 1758	43	2
Hooded crow — *Corvus cornix* Linnaeus, 1758	50	1
Common raven — *Corvus corax* Linnaeus, 1758	1	0
Rook — *Corvus frugilegus* Linnaeus, 1758	79	0
Eurasian magpie — *Pica pica* Linnaeus, 1758	22	0
Western jackdaw — *Coloeus monedula* Linnaeus, 1758	2	0
Eurasian jay — *Garrulus glandarius* Linnaeus, 1758	6	0
Eurasian tree sparrow — *Passer montanus* Linnaeus, 1758	3	0
House sparrow — *Passer domesticus* Linnaeus, 1758	3	0
Great tit — *Parus major* Linnaeus, 1758	7	0
Red-backed shrike — *Lanius collurio* Linnaeus, 1758	1	0

End of the Table 2

Species	Amount of studied specimens	Amount of positive specimens
Song thrush — *Turdus philomelos* Brehm, 1831	2	0
American robin — *Turdus migratorius* Linnaeus, 1766	1	0
Common blackbird — *Turdus merula* Linnaeus, 1758	2	0
Fieldfare — *Turdus pilaris* Linnaeus, 1758	7	0
Garden warbler — *Sylvia borin* Boddaert, 1783	1	0
Common redstart — *Phoenicurus phoenicurus* Linnaeus, 1758	1	0
European greenfinch — *Chloris chloris* Linnaeus, 1758	1	0
Bohemian waxwing — *Bombycilla garrulus* Linnaeus, 1758	1	0
Barn swallow — *Hirundo rustica* Linnaeus, 1758	1	0
Yellowhammer — *Emberiza citrinella* Linnaeus, 1758	1	0
European robin — *Erithacus rubecula* Linnaeus, 1758	5	0
Eurasian chaffinch — *Fringilla coelebs* Linnaeus, 1758	1	0
Blyth's reed warbler — *Acrocephalus dumetorum* Blyth, 1849	2	0
Arctic warbler — *Phylloscopus borealis* Blasius, 1858	2	0
Eurasian wren — *Troglodytes troglodytes* Linnaeus, 1758	1	0
Common grasshopper warbler — *Locustella naevia* Boddaert, 1783	1	1
Spotted flycatcher — *Muscicapa striata* Pallas, 1764	1	0
Barred warbler — *Curruca nisoria* Bechstein, 1795	1	0
Common swift — *Apus apus* Linnaeus, 1758	2	0
Black woodpecker — *Dryocopus martius* Linnaeus, 1758	1	0
Great spotted woodpecker — *Dendrocopos major* Linnaeus, 1758	2	0
European Nightjar — *Caprimulgus europaeus* Linnaeus, 1758	1	0
Ural owl — *Strix uralensis* Pallas, 1771	1	0
Tawny owl — *Strix aluco* Linnaeus, 1758	4	0
Red-footed falcon — *Falco vespertinus* Linnaeus, 1766	1	0
Western marsh harrier — *Circus aeruginosus* Linnaeus, 1758	1	0
Black kite — *Milvus migrans* Boddaert, 1783	1	0
Common buzzard — *Buteo buteo* Linnaeus, 1758	1	0
Total	684	6
Small mammals		
Bank vole — *Myodes glareolusu* Schreber, 1780	186	0
East European vole — *Microtus majori* Satunin, 1907	1	0
Common vole — *Microtus arvalis* Pallas, 1779	40	0
Feldmäuse – *Microtus* Schrank, 1798, sp.	7	0
European water vole — *Arvicola amphibius* Linnaeus, 1758	1	0
House mouse — *Mus musculus* Linnaeus, 1758	8	0
Yellow-necked mouse — *Apodemus flavicollis* Melchior, 1834	9	0
Ural field mouse — *Sylvaemus uralensis* Pallas, 1811	134	0
Striped field mouse — *Apodemus agrarius* Pallas, 1771	35	0
Short-tailed field vole — *Microtus agrestis* Linnaeus, 1761	1	0
Brown rat — *Rattus norvegicus* Berkenhout, 1769	5	0
European mole — *Talpa europaea* Linnaeus, 1758	1	0
Common shrew — *Sorex araneus* Linnaeus, 1758	27	0
Total	455	0
Amphibians		
Marsh frog — Pelophylax ridibundus Pallas, 1771	45	0

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Table 3. Results WNV RNA testing in arthropod species in 2024

Species	Amount of specimens	Amount of studied samples	Amount of positive samples
Blood-sucking mosquitoes			
Anopheles algeriensis Theobald, 1903	172	7	0
Anopheles claviger Meigen, 1804	695	36	0
Anopheles hyrcanus Pallas, 1771	4096	146	0
к. Anopheles maculipennis Meigen, 1818	38 292	1577	2
Anopheles plumbeus Stephens, 1828	8	2	0
Aedes albopictus Skuse, 1895	30	1	0
Aedes annulipes Meigen, 1830	111	6	0
Aedes behningi Martini, 1926	201	11	0
Aedes cantans Meigen, 1818	4687	164	0
Aedes caspius Pallas, 1771	2865	109	0
Aedes cataphylla Dyar, 1916	204	8	0
Aedes cinereus Meigen, 1818	2066	80	1
Aedes communis De Geer, 1776	964	36	0
Aedes cyprius Ludlow, 1920	5	1	0
Aedes dorsalis Meigen, 1830	878	34	0
Aedes excrucians Walker, 1856	709	25	0
Aedes flavescens Muller, 1764	1014	52	0
Aedes geniculatus Olivier, 1791	214	11	0
Aedes intrudens Dyar, 1919	380	14	0
Aedes nigrinus Eckstein, 1918	15	1	0
Aedes pulcritarsis Rondani, 1872	83	4	0
Aedes punctor Kirby, 1837	132	5	0
Aedes riparius Dyar et Knab, 1907	63	3	0
Aedes sticticus Meigen, 1838	3974	143	0
Aedes subdiversus Martini, 1926	1	1	0
Aedes vexans Meigen, 1830	8344	293	0
Culex modestus Ficalbi, 1890	5197	188	2
Culex pipiens Linnaeus, 1758	13 428	477	11
Culiseta alaskaensis Ludlow, 1906	322	24	0
Culiseta annulata Schrank, 1776	480	46	0
Culiseta longiareolata Macquart, 1838	59	6	0
Coquillettidia richiardii Ficalbi, 1889	3573	146	2
Uranotaenia unguiculata Edwards, 1913	176	8	6
Total	93 438	3665	24
Ixodid ticks			
Dermacentor marginatus Sulzer, 1776	96	13	0
Dermacentor niveus Neumann, 1897	33	3	0
Dermacentor reticulatus Fabricius, 1794	1688	193	0
Haemaphysalis punctata Canestrini and Fanzago, 1878	9	1	0
Hyalomma detritum Schulze, 1919	20	1	0
Hyalomma marginatum Koch, 1844	197	50	2
Hyalomma scupense Schulze, 1919	181	39	0
Ixodes persulcatus Schulze, 1930	960	86	0

End of the Table 3

Species	Amount of specimens	Amount of studied samples	Amount of positive samples
Ixodes ricinus Linnaeus, 1758	895	77	0
Rhipicephalus annulatus Say, 1821	342	64	0
Rhipicephalus niveus Yamazaki (1919)	15	2	0
Rhipicephalus rossicus Yakimov et Kohl-Yakimova, 1911	169	18	1
Rhipicephalus sanguineus Latreille, 1806	66	9	0
Rhipicephalus turanicus Pomerantsev 1936	20	2	0
Rhipicephalus pumilio Schulze, 1935	118	12	0
Total	4809	570	3

air temperature readings. In 2024, averaged anomalies were significant across most of Russia in April, throughout the summer months, and in September, which contributed to an increase in the replication rate of the pathogen, accelerated the development stages of vectors, and prolonged the stay of migratory birds in their breeding areas.

When examining the material from birds, all positive samples were found only in the Volgograd region. Among them, there are sedentary birds (gray crow) and migratory birds (common cricket, great cormorants, gray herons). A fallen common cricket was discovered by us in the area of high-rise buildings in the center of Volgograd in mid-August during the period of maximum activity of the pathogen and *Culex* mosquitoes. At the same time, over the many years of studying WNF in southern Russia, official epizootics among birds with fatal outcomes (in Volgograd and Astrakhan regions) have not been recorded. This fact was explained by the possible adaptation of local bird populations as a result of long-term interaction with the pathogen population [15]. Confirmation of the etiological role of the WNF pathogen in the occurrence of fatal disease in birds in the territory of the old disease focus in the 2024 season suggests that active monitoring for the morbidity of wild and synanthropic birds, as well as targeted examination for the presence of WNV markers in deceased individuals in accordance with regulatory documents, is not being carried out[2].

The remaining birds with detected WNV RNA were captured in fish farming ponds and lakes of the Volga-Akhtuba floodplain in the Volgograd region from September 28 to November 3. Great cormorants and gray herons use these biotopes as stops for rest and feeding during the autumn migration to wintering grounds, while resident gray crows are attracted to these water bodies due to the constant presence of food remnants from other birds on their shores.

Markers of WNV in Russia have been found in mosquitoes of 19 species over the entire observation period [16–18]. According to the results of our research in 2024, mosquitoes of 6 species tested positive for the presence of WNV RNA. Among the positive findings, *C. pipiens* accounted for 45.8%, *U. unguiculata* for 25.0%, *C. richiardii*, *Cx. modestus* and *A. maculipennis* mosquitoes for 8.3% each, and *Ae. cinereus* for 4.2%.

The level of individual infection rate in mosquitoes feeding on birds and mammals, including humans, was 0.08% for *C. pipiens*, 0.06% for *C. richiardii*, 0.04% each for *Cx. modestus* and *Ae. cinereus*, and 0.006% for *An. maculipennis*. This indicator for *Uranotaenia unguiculata* mosquitoes, whose main hosts are frogs — carriers of the lineage 4 WNV — reached 6.3%. The pathogenicity of lineage 4 WNV for humans remains unproven.

All *C. pipiens*, *An. maculipennis* and *C. richiardii* mosquitoes in which WNV markers were detected were collected in populated areas. *Ae. cinereus* and *Cx. modestus* mosquitoes were caught on the shores of water bodies in areas where waterfowl concentrate. This indicates a high risk of WNV infection for the population in both urbanized and natural habitats.

Territorially, as expected, the maximum number (20) of positive findings from mosquitoes was identified in the SFD: 8 in Astrakhan region, 10 in Volgograd region, and 1 each in Rostov region and Krasnodar Krai. In the VFD, WNV markers were found in mosquitoes: in 2 samples from the Republic of Mordovia and 1 from the Kirov region. Out of 97 samples collected in the Southern UFD in the Chelyabinsk region, the pathogen was found in 1 sample from *C. modestus* thermophilic mosquitoes, which reach high numbers only in the Southern UFD. In central Russia, however, they are usually found in small numbers and not in all regions. And although the average summer temperatures in the Southern Urals are 2.0–3.5°C lower than in the CFD as a whole, the presence of one of the main carriers of WNV in Europe in this area and the detection of the pathogen RNA from it indicate a sufficiently high risk of infection for the population in the Chelyabinsk region.

[2] Paragraphs 5.18, 5.5, 8.5.1 Epidemiological surveillance, laboratory diagnostics and prevention of West Nile fever. MU 3.1/4.2.4063-24. Moscow; 2024. 46 p. (In Russ.)

In the other surveyed regions, infected carriers of WNV were not found, which does not rule out the presence of WNV foci and requires conducting repeated studies with the selection of other biotopes for field sample collection.

Positive samples for the presence of WNV RNA from ixodid ticks were found only in the SFD: 2 from *H. marginatum* in the Volgograd region and 1 from *Rhipicephalus rossicus* in the Rostov region. Their individual infection rates were 1.0% and 1.8%, respectively.

The detection of WNV RNA in mid-April from ticks and in June from mosquitoes and ticks indicates an early activation of the epizootic process in 2024. Moreover, the April findings may also indicate the preservation of WNV in ticks during the winter period.

The established combined presence of lineages 1 and 2 WNV in the Rostov region is of scientific interest.

Conclusion

In southern Russia, the enzootic circulation of WNV in 2024 has been confirmed in the territories of Volgograd, Astrakhan, Rostov regions and Krasnodar Krai. The beginning of the epizootic process of WNF was registered in these territories (with the exception of Astrakhan region) during the spring–early summer period, which was a precursor to possible epidemiological instability. The presence of positive findings in the Republic of Mordovia, Kirov and Chelyabinsk regions confirms the circulation of the pathogen in the territories of the VFD and the UFD. Information on the spread of WNV in Russia has been supplemented with data from three new regions, and the Kirov region was the northernmost point where the pathogen RNA was detected in field samples in our study.

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Analysis of HIV-1 genetic variants and drug resistance among men with high-risk sexual behavior, Cuban citizens, living in Moscow in 2022–2024

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Abstract

Introduction. The characteristics of the HIV epidemic in Cuba are comparable to those of the HIV epidemic in Russia. Migration between Cuba and Russia can affect HIV-1 genetic diversity and drug resistance in Russia. The vulnerable group of men with risk sexual behavior including men who have sex with men (MSM) and commercial sex workers (CSW) deserves special attention. The **aim** of our study was the analysis of HIV-1 genetic variants and HIV-1 drug resistance in blood plasma samples obtained from MSM migrants from Cuba living in Moscow.

Materials and methods. A collection of blood plasma samples, epidemiological and clinical information was collected from MSM patients — migrants from Cuba, nucleotide sequences of the HIV-1 genome were obtained. HIV-1 genotyping, cluster analysis and analysis of drug resistance (DR) were carried out.

Results. Samples and epidemiological data obtained in 2022–2024 from 27 patients were analyzed. 24/27 patients (including 12/15 suspected of infection in Moscow and all 10 — in Cuba) harbored HIV-1 variants typical for Cuba, not Russia. This indicates that 88.89% of patients were infected by their fellow citizen. DR was detected in 9 patients (33.33%; 95% CI 15.55–51.11). The most common resistance was DR to efavirenz (EFV) and nevirapine (NVP), which was associated with *K103N, Y181C* and *P225H* mutations.

Conclusion. The migration factor should be taken into account in HIV-1 prevention and control of HIV-spreading programs in Russia, and the genetic characteristics of HIV-1 in migrants should be taken into account in effective therapy selecting.

Keywords: *HIV-1, MSM, migrants, viral variant, CRF, BG-recombinants, drug resistance, cluster*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Central Research Institute of Epidemiology (protocol No. 142, April 25, 2024).

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Оригинальное исследование
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Анализ генетических вариантов и лекарственной устойчивости ВИЧ-1 среди мужчин с рискованным сексуальным поведением, граждан Кубы, проживающих в Москве в 2022–2024 годах

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Аннотация

Введение. Эпидемия ВИЧ-инфекции на Кубе имеет свои особенности по сравнению с эпидемией в России. Миграционные потоки между Кубой и Россией способны оказать влияние на генетическое разнообразие и лекарственную устойчивость ВИЧ-1 в России. Отдельного внимания заслуживает уязвимая группа мужчин с рискованным сексуальным поведением, в частности — мужчины, практикующие секс с мужчинами (МСМ), и работники коммерческого секса.

Целью нашего исследования был анализ генетических вариантов ВИЧ-1 и лекарственной устойчивости ВИЧ-1 в образцах плазмы крови, полученных от мигрантов — МСМ с Кубы, проживающих в Москве.

Материалы и методы. Была собрана коллекция образцов плазмы крови, сопутствующая эпидемиологическая и клиническая информация от пациентов — МСМ с Кубы, получены нуклеотидные последовательности генома ВИЧ-1. Были проведены генотипический, кластерный анализ и анализ лекарственной устойчивости (ЛУ) ВИЧ-1.

Результаты. Были проанализированы образцы и эпидданные, полученные от 27 пациентов в 2022–2024 гг. Было выявлено, что 24/27 пациентов (включая 12/15, предполагающих факт инфицирования в Москве, и 10, предполагающих инфицирование на Кубе), были инфицированы вариантами ВИЧ-1, типичными для Кубы, а не для России. Это говорит об инфицировании 88,89% пациентов их согражданином. ЛУ была выявлена у 9 (33,33%; 95% ДИ 15,55–51,11) пациентов. Наиболее часто отмечалась устойчивость к эфавирензу и невирапину, что было связано с мутациями *K103N*, *Y181C* и *P225H* гена обратной транскриптазы.

Заключение. Фактор миграции должен учитываться в программах профилактики и противодействия распространению ВИЧ-инфекции в России, а генетические особенности ВИЧ-1 необходимо принимать во внимание при подборе эффективной терапии у мигрантов.

Ключевые слова: *ВИЧ-1, МСМ, мигранты, вирусный вариант, CRF, BG-рекомбинанты, лекарственная устойчивость, кластер*

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

Для цитирования: Лаповок И.А., Кириченко А.А., Шлыкова А.В., Киреев Д.Е., Попова А.А., Покровская А.В. Анализ генетических вариантов и лекарственной устойчивости ВИЧ-1 среди мужчин с рискованным сексуальным поведением, граждан Кубы, проживающих в Москве в 2022–2024 годах. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2025;102(4):425–435.
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Introduction

The relationships between Cuba and Russia have been significantly strengthened in recent years. In recent years, cooperation between the two countries has been based on the Declaration on Principles of Relations between the Russian Federation and the Republic of Cuba[1], the Memorandum on Principles of Strategic Cooperation[2] and the Joint Statement of the Presidents of the two countries[3], adopted in 1996, 2009 and 2018, respectively.

The close relationships between Russia and Cuba lead to increased migration processes between the countries. Since the spread of HIV infection is a problem in both countries, there is always a risk that HIV-1 variants circulating in the one country could be brought into the other country. The spread of these variants within certain vulnerable groups can then lead to an increase in infections, which can affect the genetic landscape of HIV infection and the drug resistance (DR) profile of a country. One such vulnerable group is men with high-risk sexual behaviors, particularly men who have sex with men (MSM) as well as commercial sex workers (CSW). Sexual contacts with citizens of other countries are typical for members of this group, which can affect the genetic diversity of HIV-1 and lead to the generation of new recombinant forms of the virus.

Cuba has seen an increase in the number of HIV infections in recent years. According to the United Nations Joint Program on HIV/AIDS (UNAIDS), the total number of people living with HIV-1 in Cuba has continued to increase in the last decade, rising from 3,100 in 2000 to 14,000 in 2011 [1] and by 44,000 in 2023[4]. Meanwhile, in recent years, Cuba has shown success diagnostic and treatment of HIV infection. A total of 33,000 (75%) people knew their HIV status in 2023. Of these, 28,000 (84.84%) were on antiretroviral therapy (ART). Finally, 24,000 (85.72%) patients on ART had virological success. Thus, Cuba is well on its way to meeting the WHO's 90–90–90 HIV strategy and has the prospect of reaching the main targets of the 95–95–95 strategy by 2030 [2].

The genetic diversity of HIV-1 in Cuba is very different from that of the Caribbean. While virus of subtype B is dominated in most countries of the region (which was associated with more than 90% of infections in the early 2000s) a large number of other HIV-1 genetic variants are actively circulating in Cuba, including recombinant forms that are spreading only on the island [3]. Thus, while back in the mid-1990s the dominant virus variant in Cuba was subtype B [4], a 2002 study showed that only 48% of patients were infected with this genetic variant [5]. In 2017, the proportion of subtype B in samples from patients over 18 years of age collected during the 1st half of 2017 was only 26.9%, and infection with various recombinant forms accounted for 59.5% of HIV infections [6].

With regard to subtype B prevalent in Cuba, it should be mentioned that this variant is genetically similar to the virus prevalent in the United States and most Western European countries, but differs from viruses prevalent in other Caribbean countries [1, 7, 8]. Some researchers attribute this to the repeated unrelated importations of subtype B from the United States, Canada and the European continent in the late 1970s [7]. Others suggest that it arrived in Cuba in the early 1990s from the United States, when, against the background of the economic crisis caused by the collapse of the USSR, the tourist business in Cuba began to develop and migration flows shifted to the United States [1].

In addition to subtype B, a recombinant form CRF19_cpx, whose genome is represented by fragments of the HIV-1 genome of subtypes D, A1, and G, is circulating in Cuba [3, 9]. CRF19_cpx was first described in Cuba in 1999. [10]. A detailed analysis revealed that CRF19_cpx is a recombinant of two viral variants: AG recombinant from Cameroon, later described as CRF37_cpx recombinant, and a subtype D virus from Gabon [9-11]. CRF19_cpx appears to have emerged in the Cuban community in the Democratic Republic of Congo (DRC) in 1966–1970, from where it entered the province of Villa Clara, Cuba in the late 1970s, i.e., before the spread of HIV-1 into Western European countries (in the late 1970s). Then there was its spreading in Havana and other provinces of Cuba [8, 10, 12]. By 2017, this genetic variant accounted for 24.1% of new HIV infections among patients over 18 years of age [6].

Also another recombinant CRF18_cpx with a complex, mosaic genome structure is widely distributed in Cuba. Apparently, it is also of African origin and could have come from either the DRC or the Central African Republic or the Republic of Cameroon or the Republic of Angola [1, 12, 13]. The genome structure of CRF18_cpx is more mosaic than that of CRF19_cpx and is represented by regions identical to viruses of subtypes A, F, G, H, K and U. In describing this genetic variant, 40 genetically related virus samples were identified, including CRF04_cpx and CRF13_cpx viruses [13].

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[1] Electronic fund of legal and normative-technical documents. Declaration on the principles of relations between the Russian Federation and the Republic of Cuba. 1996. URL: https://docs.cntd.ru/document/1902532?section=text

[2] Electronic fund of legal and normative-technical documents. Memorandum on the principles of strategic cooperation between the Russian Federation and the Republic of Cuba. 2009. URL: https://docs.cntd.ru/document/902161646?section=text

[3] Official website of the President of the Russian Federation. Joint statement of the President of the Russian Federation V.V. Putin and the Chairman of the State Council and the Council of Ministers of the Republic of Cuba M. Diaz-Canel Bermudez on common approaches to international affairs. 2018. URL: http://www.kremlin.ru/supplement/5354

[4] UNAIDS Country Fact Sheet. Cuba, 2023. URL: https://www.unaids.org/en/regionscountries/countries/cuba

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The period from the mid-1980s to the mid-1990s was marked in Cuba by the appearance of other recombinant forms, including BG recombinants, among HIV-infected people [1]. The BG recombinants circulating in Cuba have Cuban origin [3], having resulted from the recombination of HIV-1 variants of subtypes B and G that had previously circulated on the island [1]. All Cuban BG recombinants (CRF20, CRF23, CRF24) have a common origin from subtype G from Central Africa, circulating among heterosexuals, and subtype B (close to the one prevalent in the USA), circulating among MSM in Havana in the early 2000s. It is logical that BG recombinants were initially detected among MSM in Havana, and by 2003 were responsible for more than 30% of HIV infections in the Cuban capital [1, 8, 12].

This fact clearly illustrates the role of recombination as one of the drivers of HIV-1 genetic variability and makes Cuba one of the HIV-1 recombination hotspots along with Myanmar, South China, East Africa, Argentina and Brazil [8]. Moreover, recombinants generated in Cuba subsequently began to spread around the world. Thus, Cuban CRF20_BG is found in Spain and Greece [1].

Except for the recombinant forms mentioned above, other HIV-1 variants circulate in Cuba: at least 2 subtype C lineages from East and South Africa [1]. HIV-1 subtype G, the progenitor of BG recombinants, is likely has the Central African origin [1]. In addition, active circulation of HIV-1 subtype H has been noted in the province of Santiago de Cuba [12, 14]. Finally, some cases of HIV infection caused by CRF05_DF, which was previously reported in Belgium, DRC, Spain, and Costa Rica, have been identified [14].

The role of the vulnerable MSM group in the HIV epidemic in Cuba is crucial. While in 2002 just over 81% of HIV-infected men were MSM [5], in the first half of 2017 this index reached to 94.31% [6]. This vulnerable group is currently the dominant group in Cuba. The invasion of HIV-1 subtype B into the MSM group at the end of the twentieth century led to the active spreading and domination of this variant in Cuba [7]. The same factor became the basis for the increase in genetic diversity in Cuba: already in the early 2000s, Cuban BG-recombinant forms of HIV-1 began to spread among MSM [1, 8, 12].

Full-scale ART implementation began in Cuba in 2001 through the use of primarily generic nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) [6], achieving virologic success in 83% of patients in 2017 and nearly 86% in 2023. [6]. However, the use of ART has also led to an increase in DR in the country. In 2017, the prevalence of DR in ART-naïve patients to at least one drug was 29.8%, which is higher than this index in 2007–2011 (12.5%) and 2009–2012 (20.2%) [6].

In 2017, the primary DR to NRTIs was 10.6% and to NNRTIs was more than 23%. The prevalence of high-level DR to NNRTIs was associated with the frequency of K103N, G190A, and Y181C mutations [6]. Moreover, DR was significantly more frequently detected in people 18–25 years old compared to groups of older people.

There are significant differences between the genetic diversity of HIV-1 in Cuba and Russia. In Russia, since the late 1990s, HIV-1 sub-subtype A6 spreading in Russia and the former Soviet Union through injecting drug users has been dominant [15, 16]. The second most common HIV-1 variant is recombinant CRF63_02A1, which in recent years has been actively spreading in Central Asian countries and the Siberian region of Russia [15, 17].

In Russia, circulation of two variants of HIV-1 subtype B has been detected: Bwest, characteristic of Western Europe and Cuba, and Bfsu (IDU-B), which circulation was noted in the Russian Far East and which, together with sub-subtype A6, became the progenitor of CRF03_AB [15, 16, 18, 19]. The circulating of HIV-1 subtype C, CRF03_AB, and subtype G have also been observed in Russia [15].

In recent years, unique and rare AG-recombinant forms, probably imported from Central Asian countries, have been frequently detected in Russia [15].

The problem of HIV infection among MSM in Russia also has its own peculiarities, primarily due to the insufficient study of this vulnerable group. MSM were the main vulnerable group until the mid-1990s But after HIV-1 invasion in community of injecting drug users, these people became the main vulnerable group, and the contribution of MSM to the HIV epidemic in Russia became vanishingly low [15, 16, 18, 20]. This can be largely explained by the stigmatization of MSM and the lack of work on monitoring this group in Russia [18, 20]. Meanwhile, there are estimates of the probable prevalence of HIV-1 among MSM in different regions of the country — 5–25%, and UNAIDS in 2016 indicated the value of this index in Russia at 6% [18].

In recent years, the share of MSM among new HIV infections in Russia has been gradually increasing. Thus, by the end of 2020, only 2.8% of HIV cases in the country were associated with MSM. By the end of 2021, this figure increased to 3%, and by the end of 2023 — to 4.1%[5].

Bwest has historically dominated among Russian MSM, but in the recent past there has been a trend towards an increase in the proportion of HIV-1 sub-subtype A6 in this vulnerable group [15, 18], which suggests that the boundaries of this vulnerable group are blurring. In addition, the circulation in Russian MSM of BG recombinants genetically similar to viruses circulating in Spain and Portugal, but not to

[5] Federal Scientific and Methodological Center for the Prevention and Control of AIDS. URL: http://www.hivrussia.info

CRF20/23/24 viruses prevalent in Cuba, has been detected among Russian MSM [21].

Regarding the problem of HIV-1 DR in Russia, the most frequently detected resistance mutations were *K103N/S*, *G190A/S* in the same positions as for the Cuban samples. Meanwhile, instead of *Y181C*, the *K101E* substitution causing resistance to all NNRTIs is more often detected in Russian samples [22].

Taking into account the above-mentioned features of HIV infection in Cuba and Russia, as well as the role of MSM in the HIV epidemic in both countries, **the aim** of our study was to analyze HIV-1 genetic variants and viral drug resistance in blood plasma samples obtained from MSM migrants from Cuba living in Moscow, one of the most economically developed centers of Russia, where historically there is an extensive community of MSM and migrants.

Materials and methods

The collection of 27 blood plasma samples obtained from MSM Cuban citizens in period since March 2022 till June 2024 was studied. At the same time, clinical and epidemiologic data were collected and processed: age, dates of the last negative and first positive HIV test, information on the likely place and time of infection, number of sexual partners, experience with ART, as well as the stage of HIV infection. Patients were recruited for inclusion in the study in collaboration with non-profit organizations: Steps Foundation[6] and LaSky Center[7].

HIV-1 RNA concentration (viral load, VL) and CD4-lymphocyte count were determined in blood plasma samples.

The nucleotide sequences of the *pol* region (positions 2253–3353 of the reference strain HXB-2, GenBank number K03455) encoding HIV-1 protease and reverse transcriptase fragment in the studied samples were obtained. Sequencing was performed using the AmpliSens HIV-Resist-Seq reagent kit (Central Research Institute of Epidemiology) and an Applied Biosystems genetic analyzer (Life Technologies).

A sequence analysis was performed, including preliminary determination of the genetic variant using the HIVBlast online application[8], phylogenetic analysis in the MEGA 6.0 program [23], and cluster analysis of nucleotide sequences using the ClusterPicker 1.2.3 program (genetic distance threshold of 4.5% with bootstrap support of more than 90%)[9].

For phylogenetic and cluster analyses, the obtained sample was supplemented with nucleotide sequences of Cuban patients (n = 430) from the international HIV-1 database of the Los Alamos Institute (USA)[10], described in publications devoted to the analysis of HIV-1 samples isolated in Cuba in 2007–2017 [3, 6, 10]. For the phylogenetic analysis of genomes genetically close to HIV-1 variants circulating in Russia, we used a collection of reference sequences used earlier in the analysis of HIV-1 variants circulating in Eastern Europe and Central Asia in 2010–2019 [15].

HIV-1 DR was analyzed using the web service HIVdb of the Stanford University database[11] with the determination of both resistance mutations and DR level based on the Stanford Penalty Score calculation [22, 24].

The nucleotide sequences obtained in this study were uploaded to the Russian database of HIV resistance to antiretroviral drugs, RuHIV (https://ruhiv.ru/) under accession numbers RHD10698, RHD10712, RHD10720, RHD10721, RHD10725, RHD10727, RHD10733-RHD10736, RHD10739, RHD16068, RHD16123, RHD16132, RHD17497, RHD17505, RHD17513, RHD17514, RHD20769, RHD20773, RHD20782, RHD20783, RHD20796-RHD20798, RHD20806, RHD20813.

Results

The mean VL level was 5.33 (95% CI 5.19–5.44) log copies/mL and the mean CD4-lymphocyte count was 405 (95% CI 296.34–513.34) cells/μL. For 23 (85.19%) patients, stage 2a HIV infection was determined. Another 4 patients were at stage 3a. No significant associations were found between HIV infection stage and VL value or CD4-lymphocyte count.

We analyzed the genetic variants of HIV-1 in the samples based on a search for maximally genetically similar reference sequences from the GenBank database using the HIVBlast online application. The results of the analysis are presented in the **Table**. 21/27 (77.78%) samples were genetically close to HIV-1 reference sequences from Cuba. At the same time, 12 (57.14%) of the 21 patients infected with these HIV-1 variants suggested that their infection occurred in Moscow and not in Cuba.

Two patients were infected with HIV-1 subtype C, typical of Botswana. Another 2 patients had HIV-1 subtype B, which is close to the viruses isolated in Germany: patient M151, infected with HIV-1 subtype B, presumed to have been infected in Cuba, and patient M80 — in Moscow.

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[6] Foundation for the Prevention of Socially Significant Diseases "Steps". URL: http://stepsfund.ru

[7] Low-threshold center for the prevention of HIV infection and support for people living with HIV in Moscow and the Moscow region. URL: https://lasky.ru

[8] Los Alamos National Laboratory. HIV BLAST. URL: https://www.hiv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html

[9] Leigh Brown HIV Research Group. Picking and Describing HIV Clusters in Phylogenetic Trees. URL: https://hiv.bio.ed.ac.uk/software.html

[10] Los Alamos National Laboratory. HIV databases. URL: https://www.hiv.lanl.gov

[11] Stanford HIV Drug Resistance Database. URL: https://hivdb.stanford.edu

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Results of preliminary genotyping of HIV-1 nucleotide sequences in the HIVBlast online application

Sample	Presumed place of infection	Reference sequence in HIVBlast			
		GenBank number	genetic variant	country	genetic similarity, %
M52	Moscow	MZ004274	CRF19_cpx	Cuba	98
M67	Moscow	MZ004382	CRF19_cpx	Cuba	95
M75	Moscow	MK817409	CRF20_BG	Cuba	98
M76	Moscow	MK817388	CRF20_BG	Cuba	96
M80	Moscow	MH471360	Subtype B	Germany	96
M82	Moscow	MH667011	Subtype B	Russia	98
M88	Moscow	DQ113271	CRF19_cpx	Cuba	95
M89	Moscow	MZ004339	CRF19_cpx	Cuba	96
M90	Unknown	DQ113301	Subtype B	Cuba	96
M91	Moscow	JQ585469	Subtype B	Cuba	97
M94	Moscow	DQ113060	CRF19_cpx	Cuba	96
M96	Moscow	OL792340	Sub-subtype A6	Russia	97
M151	Cuba	KJ770458	Subtype B	Germany	96
M160	Moscow	MZ004178	CRF19_cpx	Cuba	97
M169	Moscow	MK817435	CRF18_cpx	Cuba	96
M177	Moscow	AY900579	CRF24_BG	Cuba	97
M185	Cuba	KR860993	Subtype C	Botswana	95
M186	Cuba	KR860993	Subtype C	Botswana	94
M195	Moscow	JN000054	CRF20_BG	Cuba	97
M199	Cuba	JN000009	Subtype B	Cuba	97
M208	Cuba	MK817498	CRF18_cpx	Cuba	96
M209	Cuba	MK817361	Subtype B	Cuba	97
M222	Cuba	JN000021	CRF24_BG	Cuba	96
M223	Cuba	MK817465	CRF18_cpx	Cuba	96
M224	Unknown	DQ020274	CRF20_BG	Cuba	90
M232	Cuba	MK817454	CRF18_cpx	Cuba	98
M240	Cuba	DQ113256	Subtype B	Cuba	96

Only 2 patients yielded HIV-1 samples typical for Russia: one patient was infected with a sub-subtype A6 virus and another with a virus genetically similar to subtype B references from the Czech Republic and Russia, and the Russian A6 and B reference viruses were isolated from male patients with homosexual and heterosexual transmission of HIV-1 in 2019 and 2015, respectively.

The preliminary genotyping results obtained were mostly confirmed by phylogenetic analysis with HIV-1 reference nucleotide sequences isolated from Cuban patients between 2007 and 2017 (**Fig. 1**). The same set of reference sequences and genomes under study (n = 457) were subjected to cluster analysis.

We identified 3 clusters formed by the samples studied:

1) a cluster formed by 2 HIV-1 subtype C samples from epidemiologically related patients M185 and M186;

2) a cluster formed by a sample from patient M222 and reference MK817363;

3) cluster formed by sample M160 and reference sequences MZ004165 and MZ004178.

Patient M222 was CSW practicing sex under chemical drugs. He was diagnosed with HIV infection in 2013, and his presumed site of infection in the same 2013 was Cuba. Therefore, the formation of a cluster

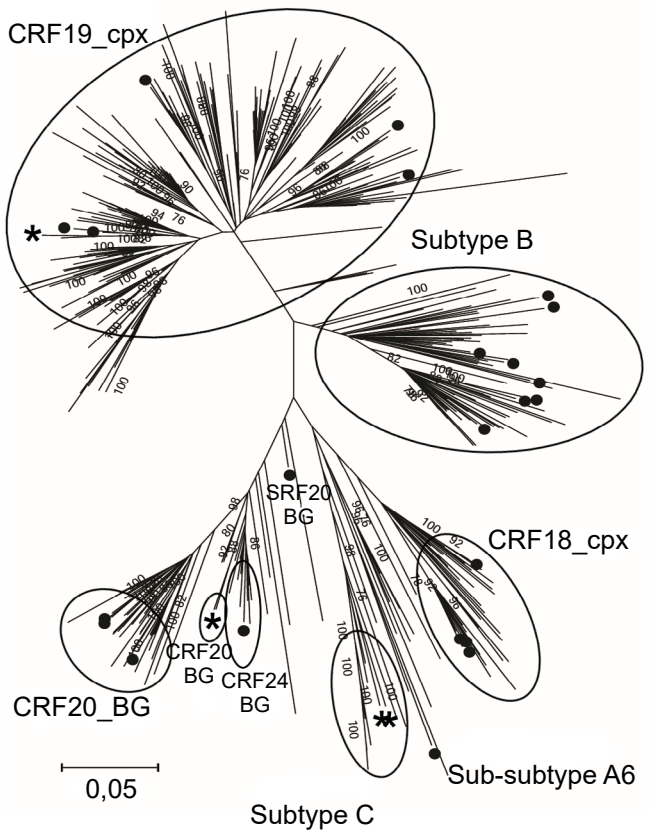

Fig. 1. Results of phylogenetic and cluster analysis of the pol gene fragment (positions 2253–3353) nucleotide sequences of the studied HIV-1 samples' collection (*n* = 27) and the comparison group (*n* = 430) of Cuban HIV-1 samples.

Phylogenetic analysis was performed by the maximum likelihood method using the GTR+G+I model of nucleotide substitutions in 500 independent constructions. Cluster analysis was performed with a genetic distance threshold of 4.5% and bootstrap support of more than 90%. Sequences of the studied collection that formed clusters are indicated by asterisks, those that did not are marked with black circles.

between M222 and MK817363, a CRF20_BG virus isolated in Cuba in 2017, is not unexpected. Interestingly, this sample was initially identified in the HIVBlast program as CRF24_BG (Table), since CRF20 and CRF24 are very close in terms of the genetic fragment studied. Cluster analysis corrected these data by assigning the investigated sample to CRF20.

At the same time, patient M160, whose sample shared a common cluster with CRF19_cpx samples MZ004165 and MZ004178 isolated in Havana in 2013 from a male and a female, respectively, was also a CSW and a transgender person. The patient was diagnosed with HIV infection in June 2023, and the estimated site and date of infection was Moscow, January 2022.

Samples from patients M82 and M96, genetically similar to Russian subtype B and sub-subtype A6 viruses, respectively, were subjected to additional phylogenetic analysis with reference sequences of HIV-1 genetic variants circulating in Russia (**Fig. 2**). Sample M96, in 82% of possible constructs, formed a common branch with the sub-subtype A6 AF413987 and AY500393 reference sequences. Sample M82, in turn, with 78% reliability formed a common branch with the Russian reference Bwest AY819715 and the main world reference of the same lineage HXB-2 K03455.

No DR to protease inhibitors was detected in any HIV-1 sample (**Fig. 3**). DR to at least one reverse transcriptase inhibitor was detected in 9 (33.33%; 95% CI 15.55–51.11) samples, and 3 (11.11%; 95% CI 0–22.97) samples (one CRF19_cpx and two CRF20_BG) were resistant to both NRTIs and NNRTIs. Typical Russian viruses from the above-mentioned samples from patients M82 and M96 did not contain DR, as well as subtype C viruses from patients M185 and M186 and HIV-1 subtype B from patient M80. The most frequently we detected DR (predominantly high level) to the NNRTIs efavirenz and nevirapine (in 29.63% of samples; 95% CI 12.41–46.85), associated with *K103N*, *Y181C* and *P225H* mutations (Fig. 3). Resistance to rilpivirine was also associated with the presence of *Y181C*, *K101E* and *E138A* mutations. Finally, high-level DR to the NNRTIs emtricitabine and lamivudine in the virus from 2 (7.41%; 95% CI 0–17.29) samples was associated with the presence of the *M184V* mutation.

Six of 9 patients infected with DR HIV-1 had no experience with therapy and 3 had past experience. Patient M222 with experience of taking efavirenz + tenofovir disoproxil + lamivudine had a virus with high level DR to efavirenz (and cross-DR to nevirapine) and lamivudine due to a combination of *M184V*, *K103N* and *P225H* mutations. Patient M208, with a history of taking Truvada 2 years prior to the study, had HIV-1 with only the *K103N* mutation causing high-level DR to efavirenz and nevirapine. The same substitution was detected in patient sample M223.

Discussion

The results of HIV-1 genetic analysis indicate that the absolute majority of 21 patients (77.78%) were infected with the virus variant typical of the patient's country of origin — Cuba. For 12 of the 15 patients who indicated Moscow as the probable place of infection, there was either infection from a Cuban citizen or a common sexual partner; or an incorrect assessment of the place of probable infection had a place. The 2 cases of infection with a typical Botswana subtype C virus most likely occurred in Cuba, as the patients assumed, bacause African variants of HIV-1 were detected in Cuba in 2013 [1]. The same is real for patient M151, who was found to have HIV infection with a subtype B virus genetically similar to the virus circulating in Germany, reflecting the link between the HIV epidemic in Cuba and Western Europe [1, 7].

Two samples, M222 (CRF20_BG) and M160 (CRF19_cpx), formed two active (expanding) clusters that also included samples isolated from Cubans in a

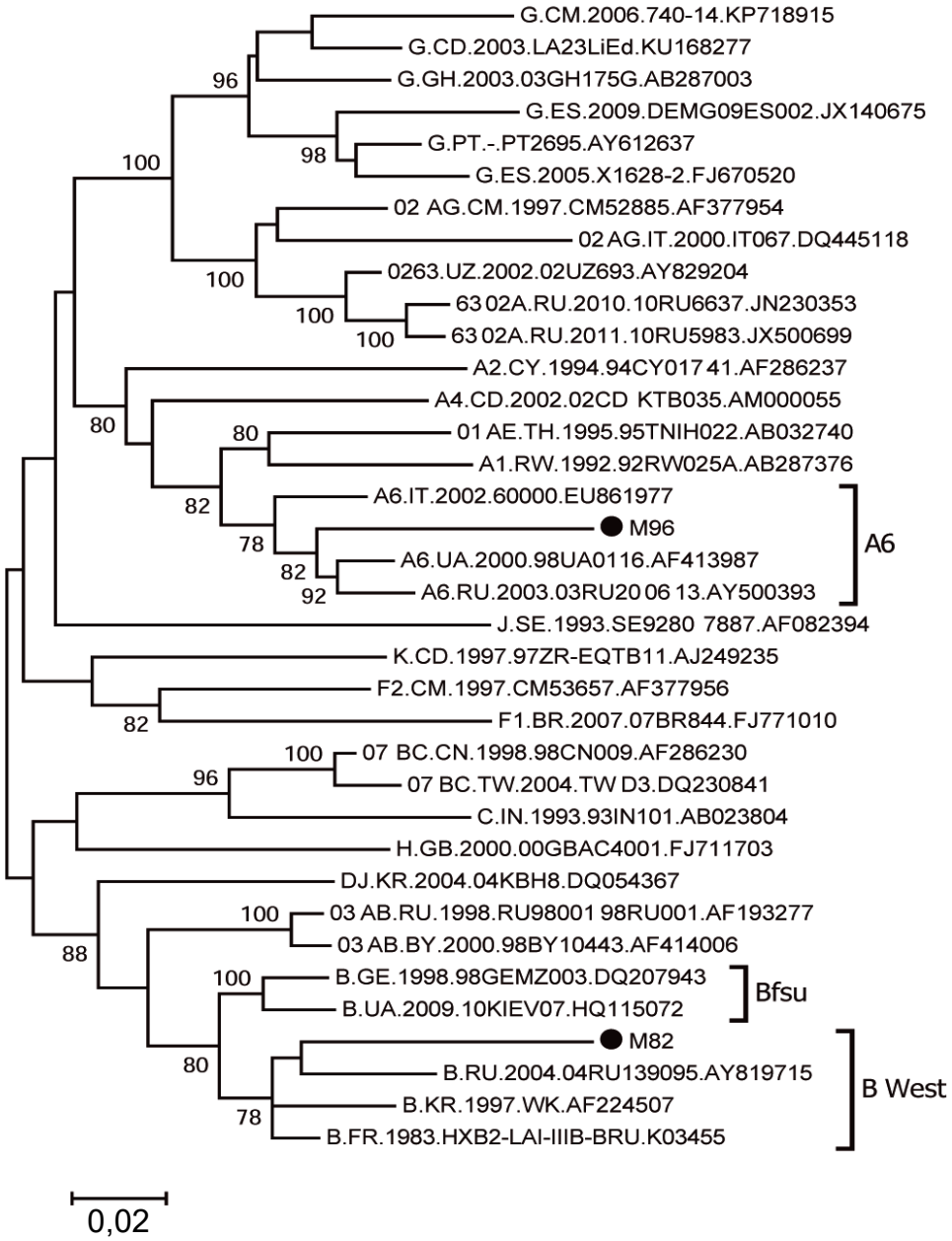

Fig. 2. Results of phylogenetic analysis of the *pol* gene fragment (positions 2253–3353) nucleotide sequences of HIV-1 samples from patients M82 and M96, presumably belonging to genetic variants circulating in Russia.

Phylogenetic analysis was performed using the maximum likelihood method with the HKY+G model of nucleotide substitutions and bootstrap support of 500. Sequences of M82 and M96 are indicated by black circles. Sub-branches formed by viruses of sub-subtype A6 and genetic variants Bwest and Bfsu are highlighted by frames.

different time period (a difference of 4 and 10 years, respectively). This suggests continued circulation and further spread of these viral genetic lineages worldwide.

Thus, a total of 24 (88.88%) of the Cuban nationals MSM we studied living in Moscow were probably infected either in their home country or from a fellow citizen. Only 3 patients could have been infected in Russia:

- M82 and M96 patients infected with HIV-1 typical for the territory of Russia;
- patient M80 infected with a subtype B virus genetically close to the strain circulating in Germany. Meanwhile, the circulation of Western European variants of HIV-1 is typical for the vulnerable MSM group in Russia [18, 20, 21].

Our data on the incidence of DR have low statistical reliability due to the small size of sample collection (n = 27), which does not allow reliable comparison of our results with published data on HIV-1 DR in Cuba. However, we detected DR in 9 virus samples, 3 of

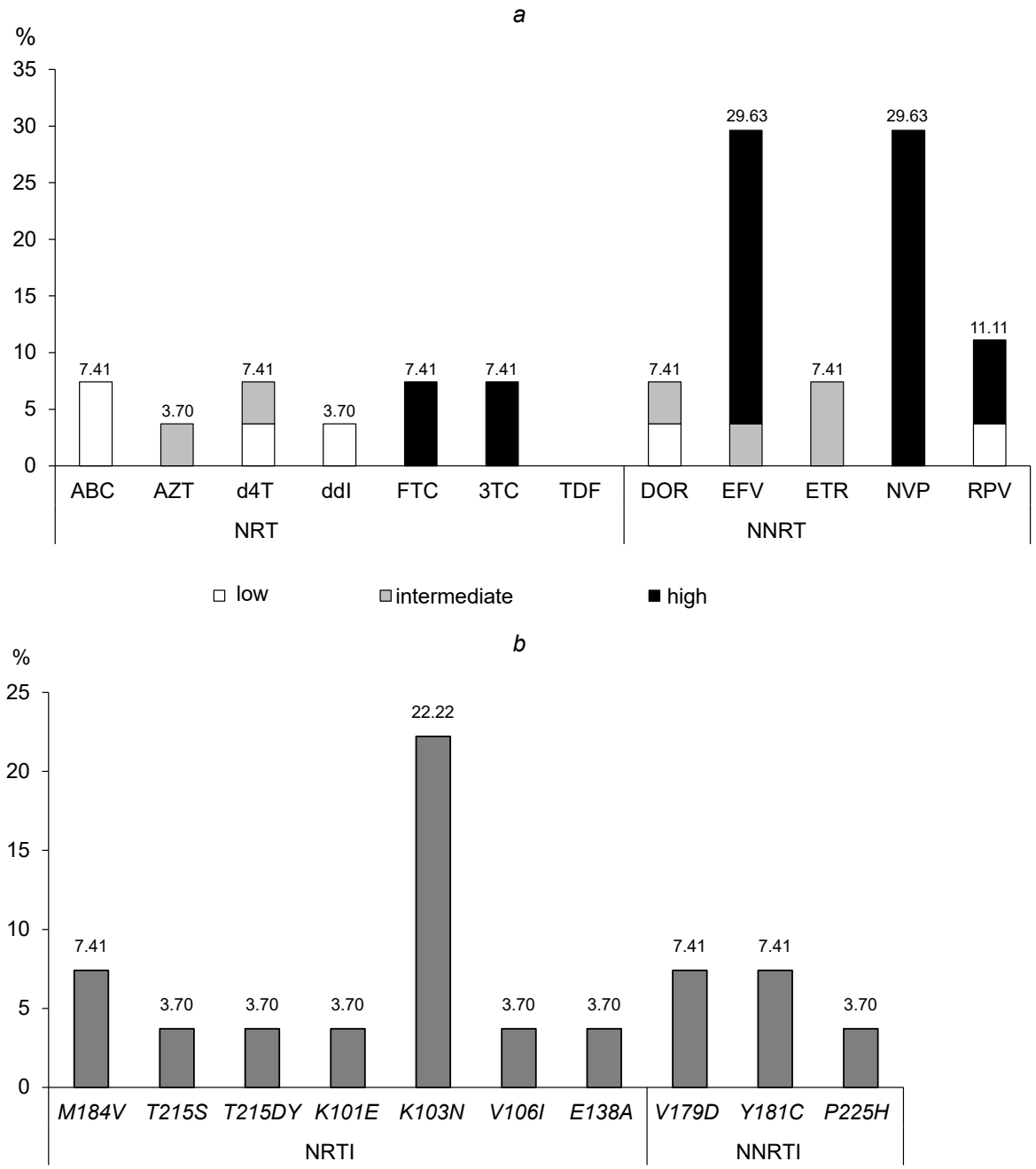

Fig. 3. Results of the analysis of occurrence frequency for drug resistance (*a*) and drug resistance mutations to HIV-1 inhibitors of the NRTI and NNRTI classes (*b*).

ABC — abacavir; AZT — zidovudine; d4T — stavudine; ddI — didanosine; FTC — emtricitabine; 3TC — lamivudine; TDF — tenofovir disoproxil; DOR — doravirine; EFV — efavirenz; ETR — etravirine; NVP — nevirapine; RPV — rilpivirine. The analysis was carried out using the HIVdb online application, the degree of resistance was determined based on the calculation of the Stanford Penalty Score.

which were obtained from patients with previous therapy experience. The most frequently detected high-level DR to efavirenz and nevirapine is a trend in recent years in Russia and low- and middle-income countries [22, 24]. The presence of HIV-1 with DR in 6 (25%; 95% CI 8.67–41.33) of 24 patients, who probably received HIV-1 from Cuban citizens, is a consequence of the problem of DR spread in Cuba [6]. Moreover, the use of effective therapeutic regimens may be the key to virologic success even in the face of HIV-1 resistance to efavirenz and nevirapine.

Conclusion

Our results suggest a contribution of migration from Cuba to HIV-1 genetic diversity among MSM in Moscow in recent years and its impact on the spread of HIV-1 DR in this vulnerable group. However, reliable data on the extent of such influence can only be

obtained by studying a wider collection of MSM group patients. The HIV-1 DR profile in the studied collection was close to published data on DR in Cuba as a whole. Thus, the migration factor should be taken into account in programs aimong to prevent and counteract the spread of HIV infection in Russia, and effective treatment of patients with HIV-1 DR variants requires the selection of an effective therapy regimen.

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Etiological structure of acute intestinal infections based on the results of exterritorial monitoring

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Abstract

Introduction. Acute intestinal infections cause high morbidity and mortality, especially in Africa and Southeast Asia, where millions of children under the age of five die every year. The Republic of Guinea urgently requires large-scale research aimed at studying the causes of diarrheal diseases, necessary for the development of effective preventive measures to ensure the preservation of the health of its population.

Aim. To conduct a analysis of the etiological structure of acute intestinal infections in the Republic of Guinea.

Materials and methods. Stool samples (n = 724) from residents of the Republic of Guinea with diarrheal syndrome were studied by real-time PCR with two reagent kits: 1) AmpliSens OKI screen-FL for the detection of DNA (RNA) of microorganisms *Shigella* spp./EIEC, *Salmonella* spp., *Campylobacter* spp., *Adenovirus*, *Rotavirus*, *Norovirus* and *Astrovirus*; 2) AmpliSens Escherichiosis-FL for the detection of DNA of diarrheagenic *Escherichia coli* (DEC) of five pathogroups: EPEC, EHEC, ETEC, EIEC, EAgEC.

Results. In the period 2019-2022, 66.2% of the examined children and adults revealed the presence of genetic markers of acute intestinal infections, mainly of bacterial etiology (74.1%), among which diarrheagenic *E. coli* dominated (62.4%). Genetic markers of viral pathogens were detected significantly less frequently by 25.9%, $p < 0.05$. Young children are most vulnerable to infection caused by *E. coli*. Bacterial pathogens dominate both in cases of monoinfections and in cases of mixed infection with two or more types of pathogens.

Conclusion. A study has shown that DEC is the main cause of intestinal infections in Guinea. The data obtained will become the basis for the development of an effective program for the prevention and treatment of acute intestinal infections in the republic.

Keywords: *diarrhea, acute intestinal infections, genetic determinants, PCR, Africa, Republic of Guinea*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. The study protocol was approved by the Ethics Committee of the Pasteur St. Petersburg Research Institute of Epidemiology and Microbiology (protocol No. 27 dated 02.07.2019).

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Оригинальное исследование
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Этиологическая структура острых кишечных инфекций по результатам экстерриториального мониторинга

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Аннотация

Введение. Острые кишечные инфекции (ОКИ) вызывают высокую заболеваемость и смертность, особенно в Африке и Юго-Восточной Азии, где каждый год умирают миллионы детей младше 5 лет. Республика Гвинея остро нуждается в проведении широкомасштабных исследований, направленных на изучение причин диарейных заболеваний, необходимых для разработки эффективных мер профилактики, обеспечивающих сохранение здоровья её населения.

Цель — провести анализ этиологической структуры ОКИ в Гвинейской Республике.

Материалы и методы. Пробы испражнений ($n = 724$) жителей Гвинейской Республики с диарейным синдромом изучали методом ПЦР в реальном времени с двумя наборами реагентов: 1) «АмплиСенс ОКИ скрин-FL» для выявления ДНК (РНК) микроорганизмов *Shigella* spp./EIEC, *Salmonella* spp., *Campylobacter* spp., *Adenovirus*, *Rotavirus*, *Norovirus* и *Astrovirus*; 2) «АмплиСенс Эшерихиозы-FL» для выявления ДНК диареегенных *Escherichia coli* (DEC) 5 патогрупп: EPEC, EHEC, ETEC, EIEC, EAgEC.

Результаты. В 2019–2022 гг. у 66,2% обследованных детей и взрослых обнаружены генетические детерминанты ОКИ, преимущественно бактериальной этиологии (74,1%), среди которых доминировали диареегенные *E. coli* (62,4%). Генетические маркеры вирусных патогенов выявлялись значительно реже — 25,9% ($p < 0,05$). Дети раннего возраста наиболее уязвимы перед инфекцией, вызванной DEC. Бактериальные возбудители доминируют как в случаях моноинфекций, так и при сочетанном заражении двумя и более видами патогенов.

Заключение. Исследование показало, что DEC являются основной причиной ОКИ в Гвинее. Полученные данные станут основой для разработки эффективной программы профилактики и лечения ОКИ в республике.

Ключевые слова: *диарейный синдром, острые кишечные инфекции, генетические детерминанты, полимеразная цепная реакция, Африка, Гвинейская Республика*

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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

Acute intestinal infections are a global health problem, leading to high morbidity and mortality rates in many countries [1]. Approximately 1.6 million people die from diarrhea worldwide each year, with the vast majority of cases occurring in developing countries [2]. Diarrheal diseases are the cause of 15% of deaths in young children, with approximately 80% occurring in the regions of Africa and Southeast Asia [3, 4]. Despite a significant global reduction in diarrhea mortality over the past quarter-century, most African countries continue to face high prevalence rates of severe acute intestinal infections [5]. Experts estimate that by 2030, 4.4 million children under the age of 5 will die annually from infectious diseases, with 60% of cases occurring in African countries [6, 7].

For the African continent, diarrheal diseases remain a pressing threat, particularly acute in conditions

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of deep poverty [8–10]. Within the UN Millenium Declaration adopted in 2000, important goals were set aimed at combating poverty and malnutrition, ensuring universal access to clean drinking water, and significantly reducing child mortality[1]. However, current realities show that many African countries will not be able to achieve these targets on time, considering that only a third of the population has regular access to clean water [11–13]. This fact creates additional risks of diarrheal diseases spreading among a significant portion of the region's population[2].

According to data from the international Global Enterics Multi-Center Study (GEMES) project, diarrheagenic *Escherichia coli* (DEC) and *Cryptosporidium* are recognized as the most dangerous pathogens causing deaths from diarrhea in children under 5 years old in several African countries — Kenya, Mali, Mozambique and Gambia. Research conducted in 18 African countries on the burden of diarrheal diseases of *Escherichia coli* etiology showed that enteroaggregative *E. coli* (EAgEC) are a more common DEC pathogenic group (69% of cases) than enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) and enteroinvasive *E. coli* (EIEC), with prevalence ranging from 6.6% to 18.6% [2].

Unlike developed regions such as the United States and Europe, which have well-organized systems for monitoring intestinal infections, including DEC[3], most countries on the African continent face significant challenges in developing effective healthcare infrastructure and comprehensive control of acute intestinal infections.

The etiology of acute intestinal infections is diverse, which leads to differences in the epidemic process in countries with varying levels of economic development. This is the reason why identifying the key pathogens causing gastrointestinal infectious diseases is crucial for organizing microbiological monitoring within the epidemiological surveillance system and preventing infectious threats in African countries. Nowadays, Guinea has a particular necessity for large-scale research aimed at studying the etiology of acute diarrhea to develop a regional strategy for preventing diarrheal diseases, which is essential for preserving the health of the Republic population.

The aim of the study is to conduct a systematic analysis of the etiological structure of acute intestinal infections in the Republic of Guinea.

Materials and methods

The study included patients who sought medical attention at healthcare facilities with symptoms of acute intestinal infection (diarrhea, nausea, vomiting, abdominal pain, symptoms of intoxication). The study protocol was approved by the Ethics Committee of the Pasteur St. Petersburg Research Institute of Epidemiology and Microbiology (protocol No. 27 dated 02.07.2019).

Stool samples from 724 patients, including 73 children aged 1–5 years (10.1%), 130 aged 6–17 years (18.0%) and 521 adults aged 18–76 years (72.0%), were examined using polymerase chain reaction (PCR) with hybridization-fluorescence detection with two reagent kits:

1. AmpliSens OKI screen-FL for the detection and differentiation of DNA (RNA) of microorganisms of the *Shigella* spp./EIEC, *Salmonella* spp., thermophilic *Campylobacter* spp., *Adenovirus* (group F), *Rotavirus* (group A), *Norovirus* (genotype 2) and *Astrovirus*;

2. AmpliSens Escherichiosis-FL for the detection of diarrheagenic *E. coli* (DEC) of 5 pathogenic groups: EPEC, EHEC, ETEC, EIEC, EAgEC (Russian Research Institute of Epidemiology of Rospotrebnadzor, Russia).

Total DNA/RNA was extracted using the RIBO-prep reagent kit (Russian Research Institute of Epidemiology of Rospotrebnadzor, Russia). PCR was performed using thermocycler with a real-time fluorescent signal detection system (real-time PCR) CXT-1000 (Bio-Rad). All nucleic acid extraction and real-time PCR procedures used in this study were performed using appropriate positive, negative and internal control samples included in the diagnostic kits. The use of controls at all stages allowed for confirmation of the correctness and accuracy of the results obtained, eliminating the possibility of false positive or false negative conclusions.

Targeted screening for *Shigella* spp. and EIEC strains was performed on stool samples that yielded a fluorescent signal for the presence of *Shigella* spp./EIEC (AmpliSens OKI screen-FL) and EIEC (AmpliSens Escherichia coli-FL). *E. coli* were isolated using the cultural method with selective media Endo and Hektoen agar (State Research Center for Applied Microbiology and Biotechnology).

Fisher's exact test was used to assess the statistical significance of differences in mean values. The significance of the differences between the studied indicators was determined using the Mann–Whitney test. Significant differences were considered at a 95% confidence interval ($p \leq 0.05$).

The studies were conducted in the laboratory of the Russian-Guinean Research Center for Epidemiology and Prevention of Infectious Diseases as part of the research project "Study of the Etiological Structure and Molecular-Genetic Characterization of Diarrheal Pathogens in the Republic of Guinea".

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[1] United Nations Millennium Declaration. URL: https://www.un.org/ru/documents/decl_conv/declarations/summitdecl.shtml

[2] WHO. Diarrhoeal disease; 2024. URL: https://who.int/news-room/fact-sheets/detail/diarrhoeal-disease

[3] National Surveillance of Bacterial Foodborne Illness (Enteric Diseases).
URL: https://www.cdc.gov/nationalsurveillance/index.html;
Surveillance and disease data for *Escherichia coli.*
URL: https://www.ecdc.europa.eu/en/escherichia-coli-ecoli/surveillance-and-disease-data

Results

According to the combined data, between 2019 and 2022, genetic markers of viral and bacterial pathogens of acute intestinal infections were detected in stool samples from 479 (66.2%) examined individuals: in 238 (49.7%; 95% CI 45.2–54.2%) men and 241 (50.3%; 95% CI 45.9–54.8%) women ($p > 0.05$). Determinants of the pathogens of interest were more frequently detected in the age group of patients 18 years and older — 331 (69.1%; 95% CI 64.8–73.1%; $p \leq 0.05$). No significant differences were found in the detection rate of DNA/RNA of pathogens causing acute intestinal infections in samples from young children ($n = 59$; 12.3%, 95% CI 9.7–15.6%) and school-aged children ($n = 89$; 18.6%; 95% CI 15.5–22.3%) (Mann–Whitney test). No statistically significant differences ($p \leq 0.05$) were found in the detection frequency of genetic markers of acute intestinal infections of established etiology based on sex and age, both in the combined data and separately by year (**Fig. 1**).

Genetic markers of the studied pathogens were not detected in samples from 245 patients with diarrhea syndrome, thus infections of unknown etiology accounted for 33.8% on average over the multi-year period.

The results of the molecular study, by year and in total, are presented in **Table 1** and **Table 2**. Throughout all years of observation, bacterial pathogens significantly predominated over viral ones in the etiological structure of acute intestinal infections ($p \leq 0{,}05$).

DNA of bacterial pathogens was detected in 355 (74.1%; 95% CI 70.1–77.8%) samples, of which *Campylobacter* spp. accounted for 9.8% (95% CI 7.5–12.9%), *Salmonella* spp. for 1.9% (95% CI 1.0–3.5%), and DEC for 62.4% (95% CI 57.9–66.7%). Viral pathogens accounted for 25.1% (95% CI 22.2–30.0%), of which *Adenovirus* accounted for 10.2% (95% CI 7.8–13.3%), *Rotavirus* for 6.9% (95% CI 4.9–9.5%), *Norovirus* for 6.7% (95% CI 4.8–9.3%), and *Astrovirus* for 2.1% (95% CI 1.1–3.8%).

The leading pathogens throughout all years of surveillance were DEC. In the age structure, they were significantly more common in young children aged 0–5 years (91.7%; 95% CI 82.7–96.9%; $p \leq 0.05$) compared to the group of school-aged children aged 6–17 years (53.9%; 95% CI 44.9–62.8%) and adults (45.6%; 95% CI 41.3–50.0%). According to the combined data, a significant prevalence of the EAgEC pathogenic group was identified both in the structure of escherichioses (46.8%; 95% CI 41.2–52.5%) and in the overall structure of acute intestinal infections (29.3%; 95% CI 25.3–33.5%; $p \leq 0.05$). In the multi-year structure of *Escherichia coli* infections, other DEC pathogenic groups: EPEC, ETEC and EIEC accounted for 20.1%, 13.0% and 13.7%, respectively. Findings of genetic determinants of STEC were less frequent compared to other DEC pathogenic groups, with their share being 6.4% based on cumulative data.

Genetic markers of bacterial pathogens were detected significantly more often compared to viral pathogens ($p \leq 0.05$) in all years of monitoring, as well as cumulatively during the study period.

Monoinfections caused by a single type of enteric pathogen prevailed over combined etiology enteric infections in all years of surveillance (**Fig. 2**). In 290 (60.5%; 95% CI 56.1–64.8%) positive samples, genetic markers of a single pathogen were detected, with DEC being significantly more frequent at 45.9% (95% CI 43.2–56.5%) compared to other bacterial pathogens (*Campylobacter* spp. — 4.2%; 95% CI 0–6.5%; *Salmonella* spp. — 0.6%; 95% CI 0–1.6%) and viral nature 47 (9.8%) (*Adenovirus* — 3.1%; 95% CI 0–9.8%; *Astrovirus* — 0.6%; 95% CI 0–1.7%; *Norovirus* — 2.7%; 95% CI 1.6–3.4%; *Rotavirus* — 3.3%; 95% CI 0.5–8.5%). Analysis of molecular studies revealed the presence of two or more genetic markers of the investigated acute intestinal infections in 189 (39.5%; 95% CI 35.2–43.9%) samples, of which viral-bacterial were found in 74 (39.2%), bacterial-bacterial in 112 (59.3%) and viral-viral in 3 (1.6%).

Discussion

Diarrheal diseases in children and adults are a pressing issue in the Republic of Guinea. It was estab-

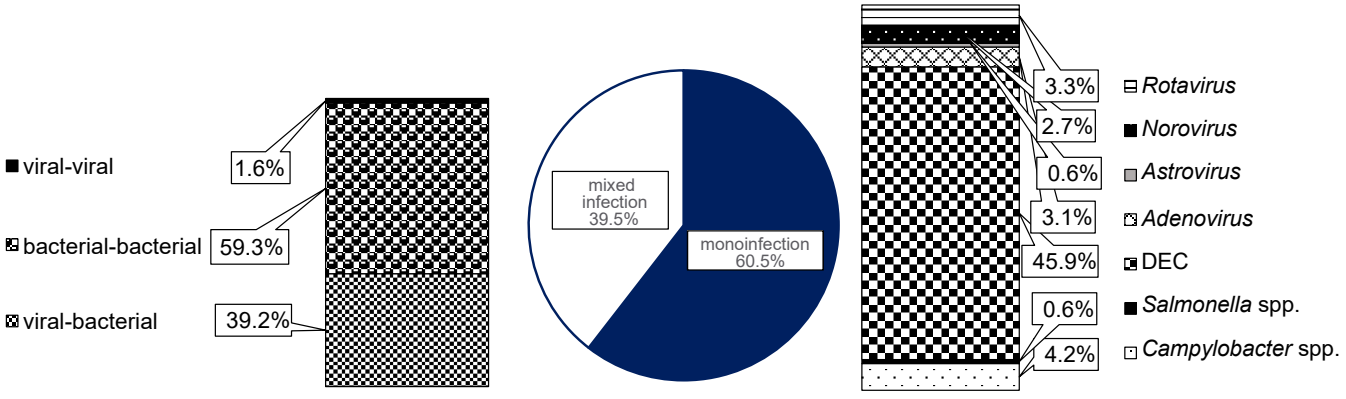

Fig. 1. Detection rates of genetic markers of acute intestinal infections in residents of the Republic of Guinea in 2019–2022.

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Table 1. Detection rates of genetic markers of enteropathogens in residents of the Republic of Guinea in 2019, 2020, 2021 and 2022

Year	Pathogen	Total		Monoinfection		Combined infections	
		n	%	*n*	%	*n*	%
2019	**Bacterial**						
	Campylobacter spp.	6	7.3	5	6.1	1	1.2
	Salmonella spp.	0	0.0	0	0.0	0	0.0
	DEC	38	46.3	36	43.9	2	2.4
	Total bacterial	44	53.7	41	50.0	3	3.7
	Viral						
	Adenovirus	30	36.6	8	9.8	22	26.8
	Astrovirus	2	2.4	1	1.2	1	1.2
	Norovirus	3	3.7	2	2.4	1	1.2
	Rotavirus	3	3.7	2	2.4	1	1.2
	Total viral	38	46.3	13	15.9	25	30.5
	Total enteropathogens	82	100.0	54	65.9	28	34.1
2020	**Bacterial**						
	Campylobacter spp.	6	5.1	1	0.8	5	4.2
	Salmonella spp.	6	5.1	1	0.8	5	4.2
	DEC	72	61.0	51	43.2	21	17.8
	Total bacterial	84	71.2	53	44.9	31	26.3
	Viral						
	Adenovirus	0	0.0	0	0.0	0	0.0
	Astrovirus	2	1.7	2	1.7	0	0.0
	Norovirus	9	7.6	4	3.4	5	4.2
	Rotavirus	23	19.5	10	8.5	13	11.0
	Total viral	34	28.8	16	13.6	18	15.3
	Total enteropathogens	118	100	69	58.5	49	41.5
2021	**Bacterial**						
	Campylobacter spp.	32	14.7	14	6.5	18	8.3
	Salmonella spp.	1	0.5	1	0.5	0	0
	DEC	142	65.4	98	45.2	44	20.3
	Total bacterial	175	80.6	113	52.1	62	28.6
	Viral						
	Adenovirus	19	8.8	7	3.2	12	5.5
	Astrovirus	2	0.9	0	0.0	2	0.9
	Norovirus	19	8.8	6	2.8	13	6.0
	Rotavirus	2	0.9	1	0.5	1	0.5
	Total viral	42	19.4	14	6.5	28	12.9
	Total enteropathogens	217	100	127	58.5	90	41.5
2022	**Bacterial**						
	Campylobacter spp.	3	4.8	0	0.0	3	4.8
	Salmonella spp.	2	3.2	1	1.6	1	1.6
	DEC	47	75.8	35	56.5	12	19.4
	Total bacterial	52	83.9	36	58.1	16	30.8
	Viral						
	Adenovirus	0	0.0	0	0.0	0	0.0
	Astrovirus	4	6.5	0	0.0	4	6.5
	Norovirus	1	1.6	1	1.6	0	0.0
	Rotavirus	5	8.1	3	4.8	2	3.2
	Total viral	10	16.1	4	6.5	6	9.7
	Total enteropathogens	62	100.0	40	64.5	22	35.5

Table 2. Detection rates of genetic markers of enteropathogens in residents of the Republic of Guinea with diarrheal syndrome in 2019–2022

Pathogen	Total		Monoinfection		Combined infections	
	n	%	*n*	%	*n*	%
Bacterial						
Campylobacter spp.	47	9.8	20	4.2	27	5.6
Salmonella spp.	9	1.9	3	0.6	6	1.3
DEC:	299	62.4	220	45.9	79	16.5
EAgEC	140	46.8	83	27.8	57	19.1
EPEC	60	20.1	35	19.7	6	0.3
ETEC	39	13.0	32	10.7	7	2.3
EIEC	41	13.7	59	11.7	1	2.0
STEC	19	6.4	11	3.7	8	2.7
Total bacterial	355	74.1	243	50.7	112	23.4
Viral						
Adenovirus	49	10.2	15	3.1	34	7.1
Astrovirus	10	2.1	3	0.6	7	1.5
Norovirus	32	6.7	13	2.7	19	4.0
Rotavirus	33	6.9	16	3.3	17	3.5
Total viral	124	25.9	47	9.8	77	16.1
Total enteropathogens	479	100.0	290	60.5	189	39.5

lished that the main pathogens of diarrheal diseases are representatives of anthroponotic infections — 363 (75.8%), of which viral (*Adenovirus, Astrovirus, Norovirus, Rotavirus*) accounts for 25.9% (95% CI 22.2–30.0%), and bacterial (EAgEC, EPEC, ETEC, EIEC) accounts for 58.5% (95% CI 54.0–62.8%). The results obtained confirm that the pathogens of anthroponotic infections are more prevalent in developing countries compared to foodborne pathogens such as *Salmonella*, *Campylobacter* and STEC in industrialized countries [14–16]. Genetic markers for *Salmonella* were identified in 9 (1.9%) cases, for *Campylobacter* in 47 (9.8%), and for STEC in 19 (6.4%). This allows for the assumption that meat and dairy products are insufficient in the

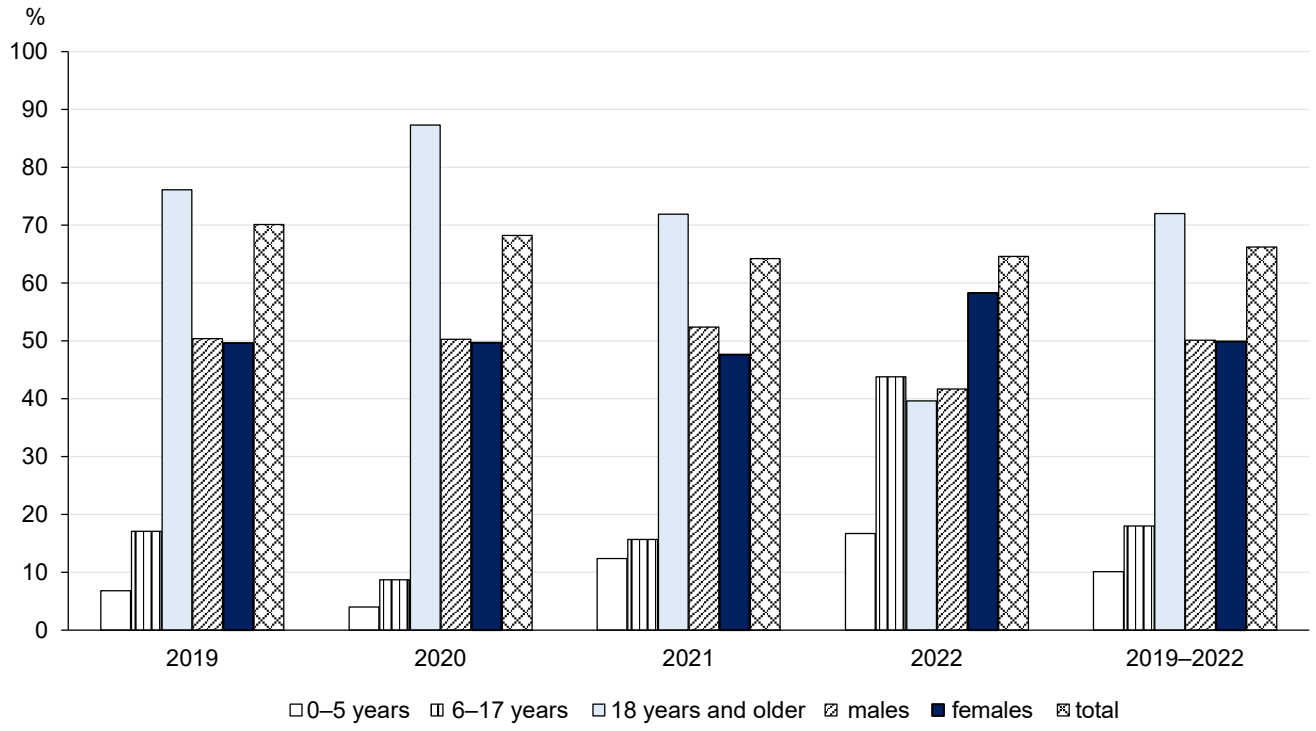

Fig. 2. Characteristics of acute intestinal infections in the Republic of Guinea in 2019–2020.

diet of the people of Guinea (children and adults), but this issue requires further study.

In the study conducted, genetic markers of Rotavirus were identified in only 33 (6.9%) patients, despite this pathogen being the most common cause of severe gastroenteritis in children in many economically developed countries, accounting for 30–72% of hospitalized patients and 4–24% of patients with mild acute gastroenteritis not requiring hospitalization. The absence of this pathogen in the etiological structure of acute intestinal infections may be related to rotavirus vaccination [17].

Monitoring revealed that DEC were the main cause of acute intestinal infections (62.4%). The use of molecular methods allowed for the assessment of the structure of *E. coli* infections and the identification of all known DEC pathogenic groups within the territory of the Republic of Guinea. According to the combined data, strains of EAgEC predominated in the structure of escherichioses in all years of surveillance, accounting for 46.8%. Studies conducted in Latin America, Asia, Africa and Eastern European countries have shown that EAgEC are more frequent causes of diarrhea in children than other bacterial pathogens [18–20]. Data obtained in the USA, Europe and Israel also indicate that EAgEC often cause diarrheal diseases in children [21]. In the United States, the incidence of *E. coli* infections caused by EAgEC is higher in young children than that of campylobacteriosis and salmonellosis [22].

Epidemiological studies in West African countries (Mali, Gambia, Burkina Faso) have detected the presence of DEC in well drinking water and in packaged sachets (water sachets, packaged water), indicating the possibility of human infection with these microorganisms. The results obtained are important for understanding the epidemiology of escherichiosis and are of interest for studying similar problems in neighboring African countries, including the Republic of Guinea [19, 23].

Overall, 290 (60.5%) residents of the Republic of Guinea were found to have genetic markers of a single enteric pathogen, and monoinfection was identified based on laboratory results. In 189 (39.5%) of the examined individuals, an association of enteropathogens was established (combined acute intestinal infections). A high prevalence of combined infections (25–53%) has been described in developing countries [24, 25]. DEC predominated in all combined infections, which is consistent with literature data [19, 20].

The use of a multiplex format in laboratory diagnostics of acute intestinal infections is currently the only highly sensitive method that allows for the establishment of the etiology of acute intestinal infections not only in the acute phase of the disease but also in asymptomatic bacterial carriage. A large-scale study conducted in Ethiopia using modern laboratory diagnostic methods showed that in 56.3% of cases, diarrheal syndrome is caused by bacterial, viral and/or parasitic pathogens, and importantly, it allowed for the identification of mixed infections in 35% of cases [26–28].

The study confirmed the relevance of diarrheagenic *E. coli* for the population of the Republic of Guinea, as well as for other African countries [2, 3, 7, 9, 19]. Laboratory diagnosis of these pathogens is only possible using molecular methods [29].

Conclusion

The etiology of acute intestinal infections in the Republic of Guinea includes bacterial and viral pathogens. The study showed that DEC were the cause of diarrheal illness in almost every other patient, which confirmed their relevance in the structure of acute intestinal infections. To reduce the burden of diarrheal diseases in Guinea, targeted epidemiological and microbiological studies are needed to identify DEC, investigate environmental contamination, including water and food, and determine risk factors. Given that diarrhea is a polyetiological disease, it is necessary to implement a comprehensive, rapid, reliable and accessible method for identifying a wide range of pathogens.

The first detailed analysis of the etiological structure of acute intestinal infections in the Republic of Guinea provides a basis for the development of evidence-based policies for the prevention and treatment of gastrointestinal infectious diseases. Such research will allow us to respond to new threats, reduce morbidity and use healthcare resources more effectively. Successful prevention of diarrheal infections requires measures such as analyzing transmission pathways, ensuring quality drinking water, increasing the population's sanitation literacy and establishing an epidemiological monitoring system. The incorporation of PCR diagnostics into routine medical practice in the Republic of Guinea will be a significant contribution to improving public health and reducing the incidence of gastrointestinal infectious diseases.

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Original Study Article
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Antimicrobial resistance of *Streptococcus pneumoniae* strains isolated from children following immunization with a 13-valent conjugated vaccine

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Abstract

Introduction. Monitoring the resistance of *Streptococcus pneumoniae* to antimicrobials is an important component of epidemiological surveillance of pneumococcal infection, addressing the challenges of improving therapeutic approaches and containing the spread of resistance at regional and national levels.

The aim of the study is to investigate pneumococcal antibiotic resistance in preschool children following immunization with a 13-valent conjugated vaccine within the national immunization schedule.

Materials and methods. From 2016 to 2022, 1250 healthy children under the age of 6 from organized groups were examined. Detection of nasopharyngeal carriage of pneumococcus was performed using the culture method, and serotype was determined by polymerase chain reaction. Antimicrobial susceptibility was investigated using the disk diffusion method, and the minimum inhibitory concentration of antibiotics in resistant isolates was determined using E-tests.

Results. 265 isolates of *S. pneumoniae* were studied, and resistance to antimicrobials was found in 36.6%. The proportion of resistant isolates was 33.8% in the early post-vaccination period (2016–2018) and 45.3% in the late period (2020–2022) (p = 0.097). Penicillin-resistant pneumococci were detected only in the early post-vaccination period, with a frequency of 11.4% (p = 0.005). The prevalence of macrolide-resistant isolates in the analyzed periods was 27.9% and 35.9% (p = 0.219), and those resistant to 3 or more classes of antibiotics were 23.4% and 17.2%, respectively (p = 0.297). The serotype composition of resistant *S. pneumoniae* has changed: vaccine serotypes 19F, 6A, 6B, etc. (83.8%) have been replaced by non-vaccine serotypes – 15AF, 23A, etc. The proportion of vaccine serotypes in the late post-vaccination period was only 10.3%.

Conclusion. Against the backdrop of child immunization against pneumococcal infection, there has been a decrease in the prevalence of pneumococci with reduced susceptibility to β-lactams. *S. pneumoniae* resistance to macrolides remains high (35.9%). Resistant pneumococcal isolates primarily belong to serogroup 15 (15AF) and serotype 23A, which are not included in the 13-valent conjugate vaccine.

Keywords: *Streptococcus pneumoniae, resistance, serotypes, asymptomatic carriers*

Ethics approval. The study was conducted with the informed consent of the patients. The study was approved by the Ethics Committee of Professor V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University (protocol No. 69, April 28, 2016) and the Independent Interdisciplinary Committee for Ethical Review of Clinical Trials (Protocol No. 1, January 17, 2020).

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Оригинальное исследование
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Резистентность к антимикробным препаратам штаммов *Streptococcus pneumoniae*, выделенных у детей на фоне иммунизации 13-валентной конъюгированной вакциной

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Аннотация

Введение. Мониторинг резистентности *Streptococcus pneumoniae* к антимикробным препаратам (АМП) является важным компонентом эпидемиологического надзора за пневмококковой инфекцией, решающим задачи по совершенствованию терапевтических подходов и сдерживанию распространения резистентности на региональном и национальном уровнях.

Цель работы — исследование антибиотикорезистентности пневмококков у детей дошкольного возраста на фоне применения 13-валентной конъюгированной вакцины в рамках национального календаря профилактических прививок.

Материалы и методы. В 2016–2022 гг. обследовано 1250 здоровых детей в возрасте до 6 лет из организованных коллективов. Выявление назофарингеального носительства пневмококка проводили культуральным методом, серотип определяли посредством полимеразной цепной реакции. Чувствительность к АМП исследовали диско-диффузионным методом, минимальную подавляющую концентрацию антибиотиков у резистентных изолятов определяли с помощью Е-тестов.

Результаты. Исследовано 265 изолятов *S. pneumoniae,* резистентность к АМП обнаружена у 36,6%. Доля резистентных изолятов составила 33,8% в раннем поствакцинальном периоде (2016–2018 гг.) и 45,3% — в позднем (2020–2022 гг.) (p = 0,097). Пенициллинрезистентные пневмококки выявлены только в раннем поствакцинальном периоде с частотой 11,4% (p = 0,005). Распространённость изолятов, устойчивых к макролидам, в анализируемых периодах составила 27,9 и 35,9% (p = 0,219), устойчивых одновременно к 3 и более классам АМП — 23,4 и 17,2% соответственно (p = 0,297). Серотиповой состав резистентных *S. pneumoniae* изменился: вакцинные сероварианты 19F, 6A, 6B и др. (83,8%) сменились невакцинными — 15AF, 23A и др. Доля вакцинных серотипов в позднем поствакцинальном периоде составила лишь 10,3%.

Заключение. На фоне иммунизации детей против пневмококковой инфекции наблюдается уменьшение распространённости пневмококков со сниженной чувствительностью к β-лактамам. Устойчивость *S. pneumoniae* к макролидам остаётся на высоком уровне (35,9%). Резистентные изоляты пневмококка в основном относятся к серогруппе 15 (15AF) и серотипу 23A, не входящим в состав 13-валентной конъюгированной вакцины.

Ключевые слова: *Streptococcus pneumoniae, резистентность, серотипы, дети-бактерионосители*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов или их законных представителей. Протокол исследования одобрен Этическим комитетом Красноярского государственного медицинского университета им. проф. В.Ф. Войно-Ясенецкого (протокол № 69/2016 от 28.04.2016) и Независимым междисциплинарным Комитетом по этической экспертизе клинических исследований (протокол № 1 от 17.01.2020).

Источник финансирования. Финансирование в части обследования детей из организованных коллективов и серотипирования выделенных культур *S. pneumoniae* осуществлялось в рамках многоцентрового международного проспективного эпидемиологического исследования особенностей пневмококковой инфекции «SAPIENS» (2016–2018 и 2020–2022 гг.), спонсором которого являлся благотворительный фонд Ростроповича–Вишневской «Во имя здоровья и будущего детей».

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

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Introduction

Streptococcus pneumoniae is a conditionally pathogenic microorganism that often colonizes the upper respiratory tract and is the pathogen of a wide range of diseases, including otitis media, sinusitis, purulent bacterial meningitis and community-acquired pneumonia. Over the past two decades, vaccination against pneumococcal infection has been implemented in many countries as part of national programs [1–4]. In Russia, immunization of children and adults in risk groups for infection has been carried out since 2014 in accordance with the National Immunization Schedule [5]. In the context of ongoing specific prevention of pneumococcal infections, research interest is focused not only on changes in the serotype and clonal landscapes of the microbial population under the influence of conjugate vaccines, but also on the dynamics of *S. pneumoniae* resistance to antimicrobials.

Pneumococcal resistance to antibiotics is characterized by a growing trend and is considered a serious threat to public health and healthcare in many countries [6]. Macrolide-resistant pneumococci are currently on the World Health Organization's list of priority bacterial pathogens (medium priority level)[1]. Previously, *S. pneumoniae* non-susceptible to penicillin were included in this list[2].

The incorporation of pneumococcal conjugate vaccines (PCV) into routine childhood immunization programs has led to a decrease in the proportion of infections caused by resistant pneumococci in many countries. This occurred due to the partial or complete elimination of *S. pneumoniae* serotypes belonging to the vaccine variants [1, 4–6]. However, in the future, against the backdrop of the ongoing serotype replacement, an increase in the proportion of resistant pneumococci belonging to non-vaccine variants of the pathogen has been noted in a number of countries. The prevalence of isolates resistant to macrolides, tetracycline, and in some countries, penicillin has sharply increased [6–8]. The serotype landscape and the prevalence of resistant *S. pneumoniae* serotypes vary significantly not only between continents and countries, but also within a single country, depending on vaccination rates, antibiotic consumption levels, and other factors [7]. In this regard, dynamic assessment of the serotype landscape and *S. pneumoniae* antimicrobial resistance, conducted both in the early and late post-vaccination periods, is one of the most important components of epidemiological surveillance of pneumococcal infections, serving as the basis for determining the strategy and tactics of specific prevention and empirical therapy for pneumococcal diseases [9]. Microbiological monitoring is particularly important in the preschool age group, where *S. pneumoniae* carriage occurs with a frequency of 24.4–54.3% [10–12], and the level, structure of resistance, as well as its molecular mechanisms are currently insufficiently studied.

Based on the above, **the aim** of our study was a comparative analysis of the structure and mechanisms of pneumococcal resistance to antimicrobials in dynamics – in the early (2016–2018) and late (2020–2022) post-vaccination periods in preschool children.

Materials and methods

1250 healthy children from organized children's groups in Krasnoyarsk were examined. The inclusion criteria were the child's regular attendance at an organized children's group (kindergarten), age 0–6 years inclusive, the presence of informed consent signed by a parent or legal guardian, and the absence of signs of infectious diseases at the time of the examination; the exclusion criterion was the presence of signs of an acute infectious disease at the time of the examination. The vaccination status was assessed based on medical documentation (form No. 112/u).

The average age of the examined children was 4.12 ± 0.97 years. Among those surveyed in 2016–2018, only 1.9% of children completed the full course of immunization (three doses, according to the national immunization schedule), 33.9% were partially vaccinated (received 1 or 2 doses of the vaccine), and 64.2% were not vaccinated. Among those surveyed in 2020–2022, those who received the full course of immunization already accounted for 68.8%, those who received 1 or 2 doses of the vaccine accounted for 26.4%, and those not vaccinated against pneumococcal infections accounted for 4.8%.

All children included in the study had nasopharyngeal swabs taken once using flocked swabs with the liquid Amies transport medium. In all cases, informed consent was obtained from the parents or legal guardians of the children included in the study. *S. pneumoniae* was cultured on Columbia agar with 5% sheep blood, nalidixic acid and colistin (ready-made agar produced by LLC Sredoff) under capnophilic conditions using Campilogaz gas generator sachets. Pneumococcal identification was performed based on colony morphology, optochin and bile tests, as well as PCR detection of the *cpsA* and *lytA* genes[3]. Serotyping was performed using multiplex

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

[2] WHO. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis. Geneva;2017.
URL: https://who.int/publications/i/item/WHO-EMP-IAU-2017.12

[3] Laboratory diagnostics of community-acquired pneumonia of pneumococcal etiology: Methodological recommendations MR 4.2.0114-16. Moscow; 2017. 64 p.

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PCR[4]. Cryopreservation of the isolated cultures was performed using Cryoinstant mix colores (Deltalab), stored at –80°C.

To determine the susceptibility of the isolates to antimicrobials, the obtained *S. pneumoniae* cultures were tested with 8 drugs: oxacillin (1 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), norfloxacin (10 μg), vancomycin (5 μg), linezolid (10 μg), and rifampicin (5 μg) using the disk diffusion method on Mueller–Hinton 2 agar (bioMerieux) supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD. Bio-Rad disks were used for this process. *S. pneumoniae* strain ATCC 49619 was used as a control. The interpretation of the results was performed in accordance with the Russian guidelines "Determination of the Susceptibility of Microorganisms to Antimicrobial Drugs" (2025 version).

To exclude β-lactam resistance mechanisms, screening with a 1 μg oxacillin disk was performed. If a zone of growth inhibition around the oxacillin disk was greater than 20 mm, the isolate was considered susceptible to all β-lactams. If the zone diameter was less than 20 mm, the isolate was considered resistant to phenoxymethylpenicillin. The diameter of the zone, 9–19 mm, indicated susceptibility to ampicillin, amoxicillin, piperacillin, cefepime, cefotaxime, ceftaroline, ceftobiprole, ceftriaxone, imipenem and meropenem. For inhibition zone diameters less than 9 mm, the minimum inhibitory concentration (MIC) of all β-lactam antibiotics was required. Accordingly, the MIC of benzylpenicillin was determined for all isolates with a zone diameter of less than 20 mm, and for cultures with a zone diameter of less than 9 mm, the MIC of amoxicillin, ceftriaxone, and imipenem was also determined. E-tests (bioMerieux) were used in this study[5]. Isolates with an MIC greater than 1 mg/L for penicillin and amoxicillin, and greater than 2 mg/L for ceftriaxone and imipenem (according to criteria for infections other than endocarditis and meningitis) were considered resistant.

An erythromycin disk (15 μg) was used to screen for macrolide resistance: isolates susceptible to it were considered susceptible to azithromycin, clarithromycin, and roxithromycin. Erythromycin-resistant isolates were considered macrolide-resistant. To detect inducible resistance to clindamycin, erythromycin and clindamycin disks were placed 12–16 mm apart, and the result was considered positive if a D-shaped zone of growth inhibition of the tested culture was present.

Pneumococci susceptible to tetracycline were considered susceptible to doxycycline and minocycline; if tetracycline resistance was detected, they were considered resistant to these antibiotics.

A 10 μg norfloxacin disk diffusion test was used to screen for resistance to fluoroquinolones. *S. pneumoniae* isolates susceptible to norfloxacin were considered susceptible to moxifloxacin and susceptible at increased exposure to levofloxacin.

Upon identifying resistance to antimicrobials in the isolate using phenotypic methods, resistance genes were detected. Erythromycin-resistant pneumococcal isolates were tested for the presence of the *ermB* and *mef* genes, which are responsible for resistance to macrolides, lincosamides, and streptogramin B, according to the method described by R.R. Reinert et al. [13]. The *tetM* gene was identified in tetracycline-resistant isolates [14]. At a benzylpenicillin MIC level exceeding 0.064 mg/L, *S. pneumoniae* isolates were tested for mutations in the genes encoding penicillin-binding proteins (PBP): *pbp1a*, *pbp2x* and *pbp2b* [15].

Statistical processing of the obtained results was performed using the Statistica v. 10.0.1011 software package. Qualitative characteristics were calculated as proportions (%), and quantitative characteristics were calculated as means and standard deviations. The distribution of the features was assessed using the Shapiro–Wilk test. If the normality of the distribution was confirmed, the Student's t-test was used for group comparisons; if not, the Mann–Whitney test (for quantitative traits) or χ^2 (for qualitative traits) was used. The level of statistical significance for the differences was considered to be $p < 0.05$ in all cases.

Results

During the study, 265 pneumococcal isolates were obtained. At the same time, between 2016 and 2018, resistance to one or more antibiotics was detected in 33.8% of the *S. pneumoniae* isolates (in 68 out of 201), while between 2020 and 2022, it was found in 45.3% (in 29 out of 64). The proportion of resistant pneumococci increased by 11.5% over 7 years ($\chi^2 = 2.76$; $p = 0.097$).

Most of the resistant isolates obtained in the early post-vaccination period were isolated from children who had not been vaccinated against pneumococcal infections, while in the late post-vaccination period, the majority of *S. pneumoniae* cultures were isolated from fully vaccinated children (**Table 1**).

Over the entire analyzed period (2016–2022), 83 isolates were obtained with a growth inhibition zone diameter around the oxacillin disk of less than 20 mm, of which 34.9% had a diameter of 9–19 mm. The MIC range of benzylpenicillin for these pneumococci was 0.016–0.750 mg/L, with an MIC_{50} level of 0.064 mg/L and an MIC_{90} of 0.19 mg/L. For isolates with a growth inhibition zone diameter of 0–8 mm ($n = 54$; 65.1%),

[4] Centers for Disease Control and Prevention. Streptococcus pneumoniae detection and serotyping using PCR. URL: https://www.cdc.gov/strep-lab/php/pneumococcus/serotyping-using-pcr.html?CDC_AAref_Val=https://www.cdc.gov/streplab/pneumococcus/resources.html (дата обращения 22.02.2025).

[5] Biomérieux. Etest. Тестирование антимикробной чувствительности. URL: https://omb.ru/upload/iblock/4c4/e_test_amoxicillinclavulanic_acid_21_xl_amoksitsillinklavulanovaya_kislota_256.pdf (дата обращения 22.02.2025).

the benzylpenicillin MIC_{50} was 1 mg/L, the MIC_{90} was 2 mg/L, with an MIC range of 0.094–8 mg/L. Thus, the proportion of penicillin-resistant isolates (according to criteria for infections other than endocarditis and meningitis) during the analyzed period was 8.7% (23 out of 265); and the proportion of those susceptible to penicillin with increased exposure was 15.5% (41 out of 265) (**Figs. 1, 2**).

When genotyping *S. pneumoniae* cultures resistant and susceptible to increased penicillin exposure, mutations in the genes encoding PBP were identified in 100% of cases. Mutations in the PBP genes were detected in 11.4% of penicillin-susceptible pneumococci and were absent in 88.6%.

Erythromycin resistance was detected in 29.8% (79 out of 265) of the isolates (**Fig. 2**), with macrolide resistance genes present in 98.7%. In 41.8% of cases, only the *ermB* gene, which encodes 23S rRNA methylation, was detected, and in 20.3%, only the macrolide efflux *mef* genes were found. The combination of these genes was detected in 38% of the isolates. Macrolide resistance genes were not detected in erythromycin-susceptible pneumococci.

S. pneumoniae resistant to clindamycin accounted for 22.6% (60 out of 265), and 3 (1.1%) isolates exhibited inducible resistance. In 61.7% of cases, the *ermB* gene was found in clindamycin-resistant isolates, and in 38.3%, a combination of the *ermB* and *mef* genes.

One-fifth — 20.8% (55 out of 265) of the *S. pneumoniae* isolates were found to be resistant to tetracycline (Fig. 2). The ribosomal protection *tetM* gene was present in 98.2% of these isolates and was not detected in tetracycline-susceptible pneumococci.

S. pneumoniae resistant to 3 or more classes of antibiotics were found in 21.9% of cases (Fig. 2).

Rifampicin resistance was detected in only 1 case. All isolates tested were susceptible to fluoroquinolones, vancomycin and linezolid.

Differences were identified in the susceptibility of *S. pneumoniae* to β-lactams depending on the observation period. In the early post-vaccination period, 23 isolates were penicillin-resistant, 37 were amoxicillin-re-

Table 1. The structure of resistant nasopharyngeal isolates of *S. pneumoniae* depending on the vaccination status of the examined children

Isolates	Early post-vaccination period (2016–2018; *n* = 68)	Late post-vaccination period (2020–2022; *n* = 29)	Significance of differences
From fully vaccinated children, *n* (%)	5 (7.35)	22 (75.86)	$\chi^2 = 47.50$; $p = 0.000$
From partially vaccinated children, *n* (%)	10 (14.71)	4 (13.79)	$\chi^2 = 0.01$; $p = 0.907$
From unvaccinated children, *n* (%)	53 (77.94)	3 (10.34)	$\chi^2 = 34.55$; $p = 0.000$

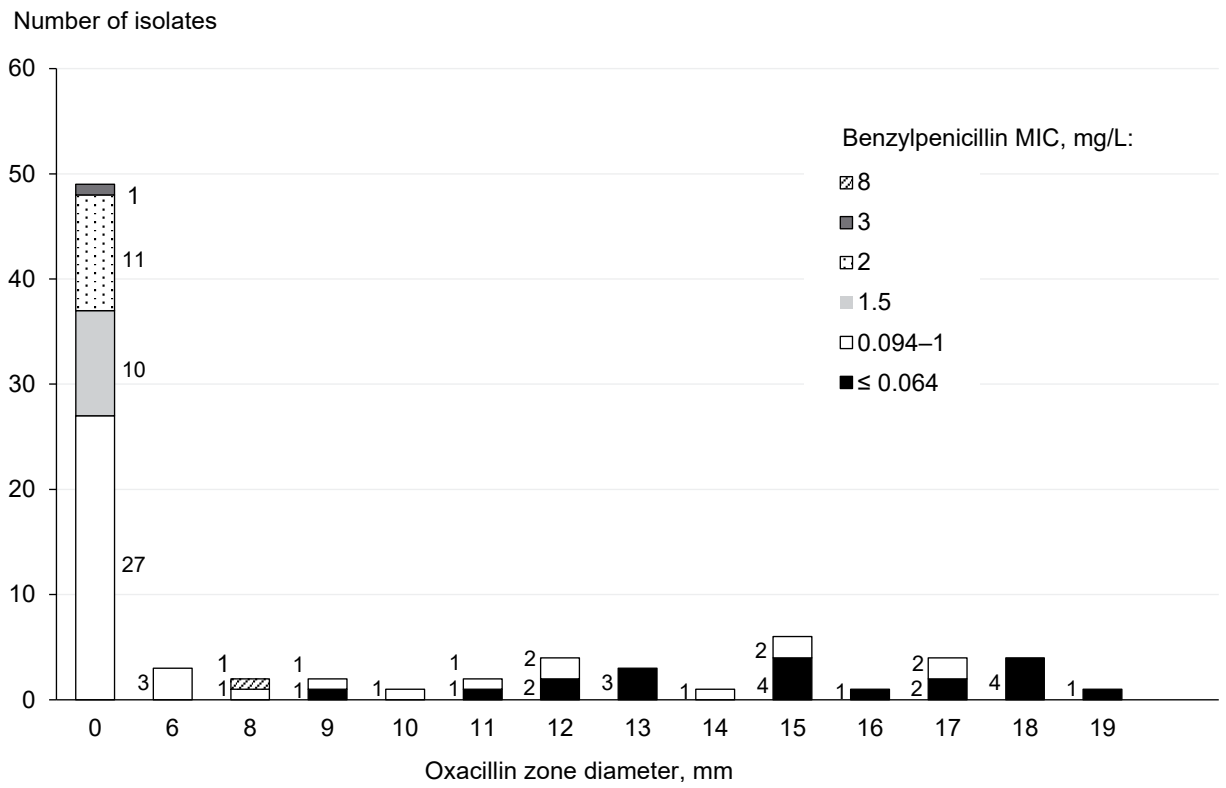

Fig. 1. MIC of benzylpenicillin in *S. pneumoniae* isolates that screened positive with 1 μg of oxacillin (*n* = 83).

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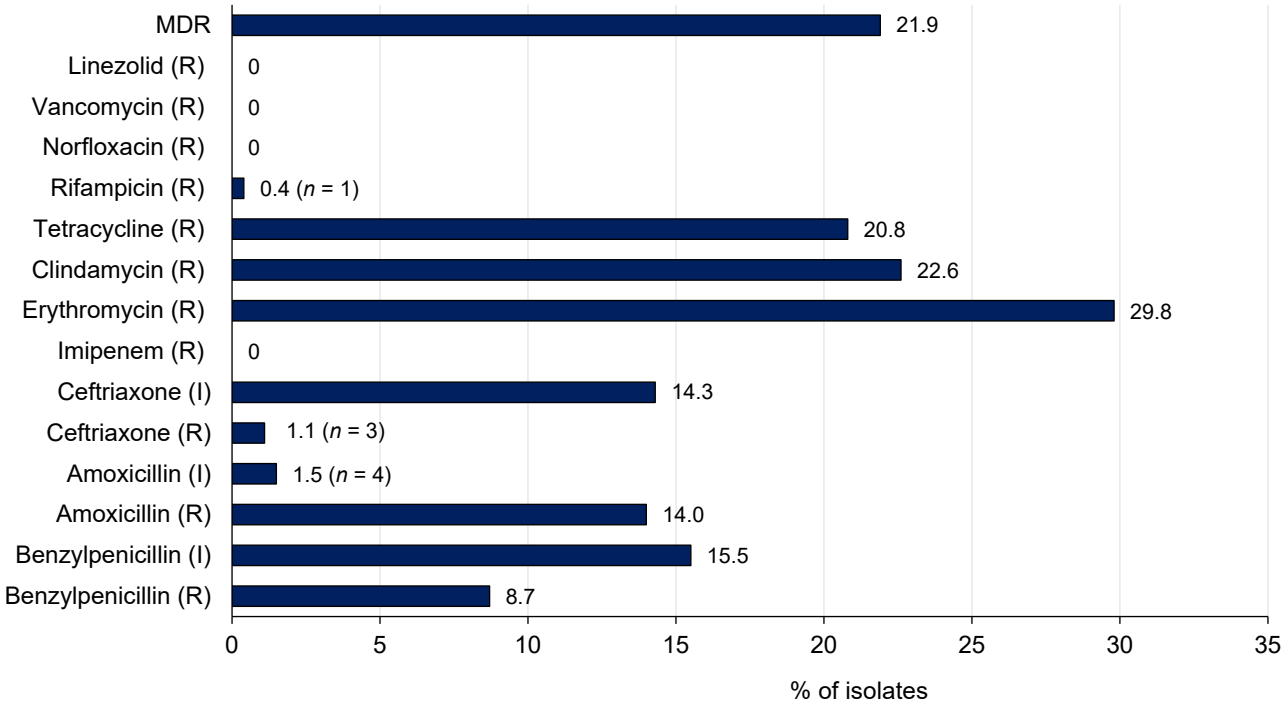

Fig. 2. Prevalence of antimicrobial resistant *S. pneumoniae* isolates.
Here and in Fig. 3: R — resistant; S — susceptible at normal dosing; I — susceptible at increased exposure.

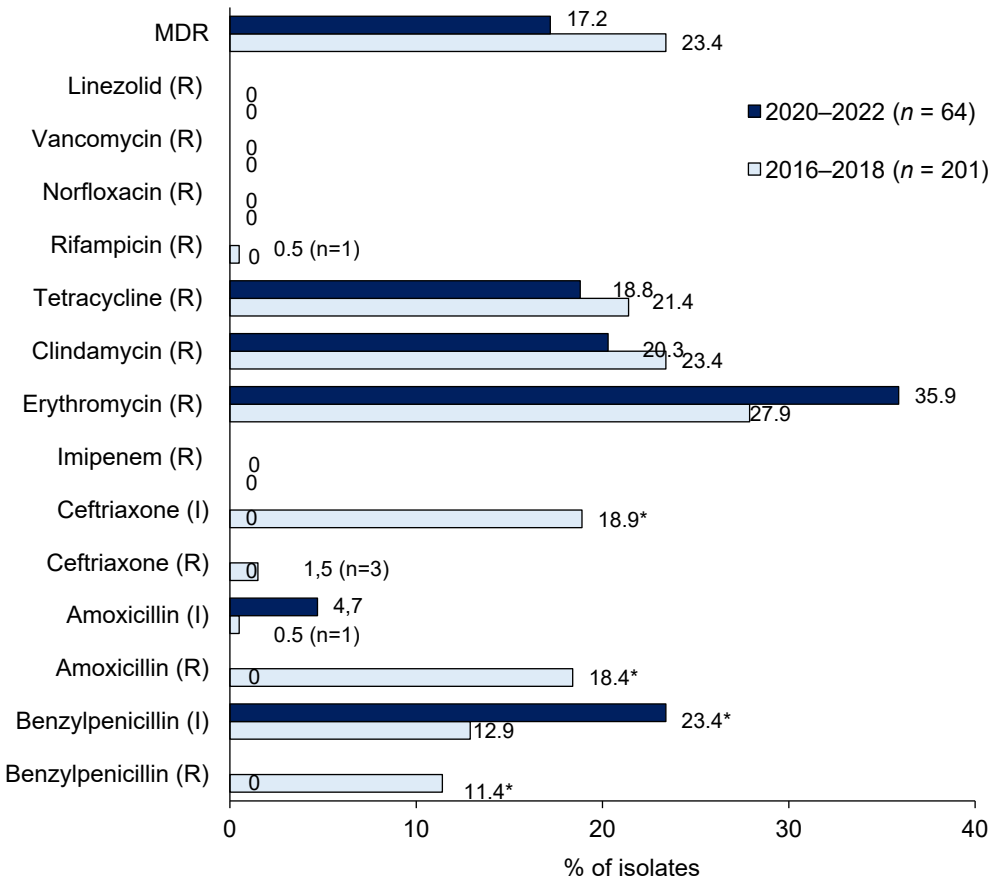

Fig. 3. Prevalence of antimicrobial resistant *S. pneumoniae* isolates.
*Statistically significant differences.

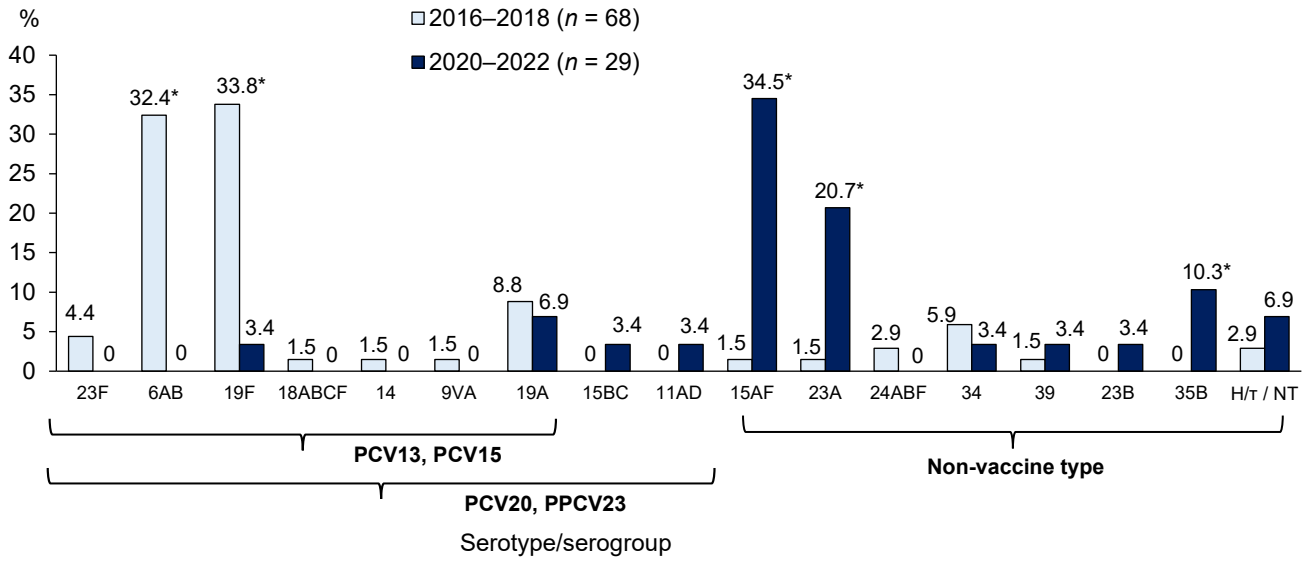

Fig. 4. Dynamics of the prevalence of *S. pneumoniae* serotypes (serogroups) resistant to antimicrobials.
N/t (non-typed) – isolates whose serotype has not been determined (not included in the standard serotyping scheme). PPSV23 is a 23-valent pneumococcal polysaccharide vaccine.*Statistically significant differences.

sistant, and 3 isolates were resistant to ceftriaxone (according to criteria for infections other than endocarditis and meningitis) (**Fig. 3**). The proportions of isolates susceptible to penicillin, amoxicillin, and ceftriaxone at increased exposure were 12.9%, 2.0% and 18.9%, respectively.

In the late post-vaccination period, isolates resistant to penicillin, amoxicillin and ceftriaxone were not detected ($\chi^2 = 8.02$; $p = 0.005$; $\chi^2 = 13.69$, $p = 0.000$; and $\chi^2 = 0.97$; $p = 0.326$, respectively) (Fig. 2). The proportions of isolates susceptible to penicillin, amoxicillin, and ceftriaxone at increased exposure were 23.4% ($\chi^2 = 4.09$; $p = 0.044$), 4.7% ($\chi^2 = 1.37$; $p = 0.242$) and 0% ($\chi^2 = 14.13$; $p = 0.000$), respectively.

The prevalence of *S. pneumoniae* resistant to macrolides, clindamycin, and tetracycline did not change significantly during the periods presented ($\chi^2 = 1.51$; $p = 0.219$; $\chi^2 = 0.26$; $p = 0.610$ and $\chi^2 = 0.21$; $p = 0.650$, respectively; Fig. 3). The proportion of pneumococci with inducible resistance to clindamycin was 1% (2 isolates) in the early post-vaccination period and 1.6% (1 isolate) in the late post-vaccination period.

The proportion of *S. pneumoniae* exhibiting resistance to 3 or more classes of antimicrobials did not differ during the observation periods ($\chi^2 = 1.09$; $p = 0.297$; Fig. 3). In the early post-vaccination period, the majority of isolates showed simultaneous resistance to β-lactams, macrolides, lincosamides and tetracyclines (69.6%), while in the late period, the phenotype of all pneumococci resistant to 3 or more classes of antimicrobials (100%) was characterized by simultaneous resistance to macrolides, lincosamides and tetracyclines.

Significant differences were found in the serotype landscape of resistant *S. pneumoniae* in the early and late post-vaccination periods (**Fig. 4**). In 2016–2018, serotypes belonging to the vaccine serotypes included in the 13-valent conjugated vaccine used were predominant: 19F ($\chi^2 = 10.07$; $p = 0.002$), 6A and 6B ($\chi^2 = 12.13$; $p = 0.000$). In 2020–2022, non-vaccine serotypes/serogroups of pneumococcus predominated: 15AF ($\chi^2 = 22.04$; $p = 0.000$), 23A ($\chi^2 = 11.22$; $p = 0.000$), and 35B ($\chi^2 = 7.25$; $p = 0.008$). During this period, *S. pneumoniae* of serogroups 11 and 15 (15BC and 11AD) were also detected, which are additionally included in the 20- and 23-valent PCV.

The resistance phenotypes of *S. pneumoniae* of different serotypes and serogroups varied: most serogroup 19 pneumococci (19F and 19A) were found to be resistant to 3 or more classes of antimicrobials, with the high proportion of penicillin-resistant isolates being noteworthy (**Table 2**). A lower prevalence of *S. pneumoniae* resistant to 3 or more classes of antimicrobials was found among members of serogroup 6 – 6A and 6B. All serotype 23A pneumococci were also resistant to 3 or more classes of antimicrobials, while penicillin resistance has not been established in representatives of this serotype.

Among pneumococci belonging to serogroup 15AF, macrolide-resistant isolates predominated. All *S. pneumoniae* serotypes 23F, 34, and 35B showed susceptibility with increased exposure to penicillin. All serotype 35B isolates were macrolide-resistant and had *mef* genes (Table 2).

Discussion

This paper presents the results of an analysis of a population of pneumococci isolated from the nasopharynx of healthy preschool children in 2016–2022, against the backdrop of PCV13 use in accordance with the Russian National Immunization Schedule. At the

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Table 2. Phenotypes and genetic mechanisms of resistance in pneumococci belonging to serotypes prevalent in the early and late post-vaccination periods

Serotype / Serogroup	Amount of isolates	Percentage of resistant isolates, % or absolute number when the amount of isolates is less than 10						Identified resistance mechanisms, % or absolute number when the amount of isolates is less than 10			
		benzylpenicillin *	amoxicillin	ceftriaxone	erythromycin	clindamycin	tetracycline	mutations in PBP genes	*ermB* gene	*mef* gene	*tet* gene
Early post-vaccination period											
23F	3	R — 0 I — 3	0	0	0	0	0	*pbp1a* + *pbp2b* — 2; *pbp1a* + *pbp2b* + *pbp2x* — 1	0	0	0
6AB	22	R — 9.1% I — 91.9%	9.1	0.0	95.5	90.1	59.1	*pbp1a* + *pbp2b* + *pbp2x* — 22.7; *pbp2x* + *pbp2b* — 68.2	95.5	22.7	95.5
19F	24	R — 70.8% I — 29.2%	95.8	8.3	100.0	83.3	54.2	*pbp1a* + *pbp2b* + *pbp2x* — 95.8; *pbp2x* — 4.2	83.3	83.3	87.5
19A	8	R — 3 I — 5	6	1	8	8	8	*pbp1a* + *pbp2b* + *pbp2x* — 8	8	8	8
34	5	R — 0 I — 5	4	0	0	0	0	*pbp1a* + *pbp2b*+ *pbp2x* — 5	0	0	0
Late post-vaccination period											
15AF	11	R — 0.0% I — 36.4%	0.0	0.0	81.8	36.4	27.3	*pbp1a* + *pbp2b* + *pbp2x* — 45.5	36.4	45.5	27.3
23A	7	R — 0 I — 4	0	0	7	7	6	*pbp2x* + *pbp2b* — 6	7	0	6
39	2	R — 0 I — 2	1	0	0	0	0	*pbp1a* + *pbp2b* + *pbp2x* — 2	0	0	0
35B	3	R — 0 I — 3	0	0	3	0	0	*pbp1a* + *pbp2b* + *pbp2x* — 3	0	3	0

Note. *For benzylpenicillin, the percentage of resistant isolates (R), as well as susceptible at increased exposure isolates (I), is indicated.

same time, resistant *S. pneumoniae* isolates obtained in 2016–2018 (the early post-vaccination period) were predominantly isolated from children who had not been vaccinated against pneumococcal infection (77.9%), while those obtained in 2020–2022 (the late post-vaccination period) were mainly isolated from children who had been fully vaccinated (three times), accounting for 75.9%. Accordingly, the proportion of fully vaccinated children also increased over the past period, from 1.9% to 68.8% ($\chi^2 = 691.47$; $p = 0.000$), indicating a significant increase in immunization coverage.

The dynamics of antimicrobial resistance in the bacterial population depend on many factors, one of which, in the case of *S. pneumoniae*, is vaccination. It has been proven that the use of conjugate vaccines leads to a decrease in the frequency and duration of pneumococcal colonization of the upper respiratory tract — primarily by the serotypes included in the vaccine [6–8]. However, data on changes in the serotype composition and resistance of *S. pneumoniae* following population immunization with conjugate vaccines are contradictory [16–21]. For instance, the results of a study conducted in Japan, along with the detection of changes in the serotype landscape and new serotypes 15A and 35B, showed a significant increase in the resistance of *S. pneumoniae* to β-lactam antibiotics — penicillin and meropenem — following the use of PCV13 [16]. The increase in resistance levels is also evidenced by a study conducted in Portugal: following the immunization of children within the national PCV13 program, the proportion of penicillin- and macrolide-resistant pneumococci increased from 9.3% and 13.4% to 20% due to the prevalence of non-vaccine serotypes 11A, 15BC, 24F, 15A and 21 [17].

The data in this study also indicate a change in the serotype composition of the resistant pneumococcal population: the serotypes that were predominant in the early post-vaccination period — 19F, 6A and 6B — have been replaced by non-vaccine variants such as 15AF, 23A and others. Accordingly, if 83.8% of resistant pneumococci belonged to the vaccine serotypes included in PCV13 in the early post-vaccination period, the proportion of such isolates was only 10.3% in the late post-vaccination period ($\chi^2 = 46.52$; $p = 0.000$).

Data on the circulation of resistant *S. pneumoniae* in Russia are mainly presented in the results of studies, including multicenter ones, involving isolates obtained from both adult and child carriers and patients with various nosological forms of pneumococcal infections [18–23].

For instance, in the study by Z.A. Alacheva et al., among isolates obtained from children in 2017–2022, the resistance level to erythromycin was 33%, to tetracycline — 26%, to trimethoprim/sulfamethoxazole –

25%, and to clindamycin — 19% [18]. Most isolates were susceptible to penicillin, and 27% were susceptible with increased exposure. Resistance to 3 or more classes of antibiotics was detected in 24.2% of pneumococci, the majority of which belonged to serotype 19F [18]. According to the multicenter SPECTRUM study, which included *S. pneumoniae* isolates obtained from adults, resistance to erythromycin in pneumococci isolated from carriers was 23.2%, and to tetracycline was 25.5%. Strains susceptible to penicillin at increased exposure were found in 16.3% of cases [19].

The results that were obtained in this study during a similar period (2016–2022) are comparable to the data presented above: the proportion of erythromycin-resistant pneumococcal isolates was 29.8%, tetracycline-resistant isolates was 20.8%, and clindamycin-resistant isolates was 22.6%. Isolates susceptible to penicillin at increased exposure accounted for 15.5%, while resistant ones made up 8.7%. Pneumococci resistant to 3 or more classes of antimicrobials were found in 21.9% of cases. At the same time, all *S. pneumoniae* isolates showing resistance, as well as susceptibility at increased exposure to penicillin, were found to have altered genes encoding PBP. In macrolide-resistant pneumococcal isolates, the *ermB* gene was detected in 41.8% of cases, the *mef* macrolide efflux genes in 20.3%, and a combination of these genes in 38%. Resistance to clindamycin was associated with the presence of the *ermB* gene in 61.7% of cases, and with a combination of the *ermB* and *mef* genes in 38.3%. Tetracycline-resistant *S. pneumoniae* were found to have the *tetM* ribosomal protection gene in 98.2% of cases.

A comparative analysis of the prevalence of resistant *S. pneumoniae* in 2016–2018 and 2020–2022 revealed the absence of isolates resistant to β-lactams — penicillin ($\chi^2 = 8.02$; $p = 0.005$), amoxicillin ($\chi^2 = 13.69$; $p = 0.000$), and ceftriaxone ($\chi^2 = 0.97$; $p = 0.326$) — in the late post-vaccination period, while in the early post-vaccination period, the proportion of pneumococci resistant to these drugs was 11.4%, 18.4%, and 1.5%, respectively. Also, in the late post-vaccination period, no isolates susceptible to ceftriaxone at increased exposure were detected, and in 2016–2018, the proportion of such isolates was 18.9% ($\chi^2 = 14.13$; $p = 0.000$). Similar data were obtained in studies by H. Dabaja-Younis et al. [21] and K. Andrejko et al. [22], which demonstrated a significant decrease in the prevalence of penicillin-resistant pneumococci in the pediatric population following PCV vaccination.

The proportion of macrolide-resistant pneumococci remained high despite immunization and tended to increase, rising from 27.9% to 35.9%. At the same time, the proportion of *S. pneumoniae* resistant to clindamycin and tetracycline among the identified resistant isolates significantly decreased – from 69.1% to 44.8% ($\chi^2 = 5.08$; $p = 0.025$) and from 63.2% to 41.4% ($\chi^2 = 3.96$; $p = 0.047$), respectively. However, the decrease was insignificant when calculated for the total pneumococcal population. The prevalence of pneumococci resistant to 3 or more classes of antibiotics among preschool children did not change significantly, being 23.4% and 17.2% in the early and late post-vaccination periods, respectively. Throughout the analyzed period, antibiotic resistance of vaccine serotypes of *S. pneumoniae* was generally higher than that of non-vaccine serotypes, which correlates with data from the PEGAS study (2015–2020) [23].

Conclusion

Following routine PCV13 immunization in children, there has been a decrease in the prevalence of pneumococci with reduced susceptibility to β-lactams: amoxicillin by 14.2% ($\chi^2 = 7.50$; $p = 0.007$) and ceftriaxone by 20.4% ($\chi^2 = 15.44$; $p = 0.000$), as well as a trend toward a decrease in the prevalence of isolates resistant to lincosamides and tetracyclines. *S. pneumoniae* resistance to macrolides remains high in the late post-vaccination period, at 35.9%.

Resistant *S. pneumoniae* isolates mainly belong to serogroup 15 (15AF) and serotype 23A, which are not included in the PCV13 used.

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Analysis of the gene structure of antiphage systems of non-toxigenic strains of *Vibrio cholerae* O1 biovar El Tor

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Abstract

Introduction. The presence and structure of antiphage systems that contribute to the resistance of cholera vibrios to lytic phages in non-toxigenic strains of *Vibrio cholerae* O1 biovar El Tor isolated in the Russian Federation and neighboring countries has not been studied.

The aim of the study is the detection and analysis of antiphage systems of non-toxigenic strains of *V. cholerae* O1 biovar El Tor.

Materials and methods. The study involved 126 non-toxigenic (*ctxAB*$^-$*tcpA*$^+$ and *ctxAB*$^-$*tcpA*$^-$) strains of *V. cholerae* O1 El Tor isolated from 1972 to 2018. DNA sequencing was performed on the MGI DNBSEQ-G50 platform. For bioinformatics analysis, the following programs were used: fastp v. 0.23, unicycler v. 0.4.7, Blast 2.16.0, MEGA X, CRISPRCasty-per and CRISPRCasFinder.

Results. Phage-inducible islands of the PLE, BREX and DISARM systems were not detected in the genome of the studied strains. It was found that 80% of *ctxAB*$^-$*tcpA*$^+$ strains contain the type I restriction-modification system, while this system was not detected in *ctxAB*$^-$*tcpA*$^-$ isolates. The genes of the CBASS system were detected in single strains of both groups. In the genome of 35 (32%) studied *ctxAB*$^-$*tcpA*$^-$ strains isolated in different regions of the Russian Federation and neighboring countries, the presence of the CRISPR–Cas system of class 1 types I (subtypes I-E, I-F, I-C) and III (subtype III-B) was established. The number of spacers in this system varied from 0 to 80 and their sequence was homologous to the protospacer regions of DNA of lytic and temperate phages, transposons, plasmids of *V. cholerae*, representatives of the genus *Vibrio* and unrelated bacteria. The presence in a number of strains of spacers homologous to the genetic material of the phage circulating in endemic territories may indicate the imported nature of these strains.

Conclusion. The heterogeneity of the studied non-toxigenic strains of *V. cholerae* O1 El Tor in the presence of antiphage systems was revealed, which expands the information on their genetic organization. In their genome, restriction-modification systems of type I (*ctxAB*$^-$*tcpA*$^+$), CBASS (*ctxAB*$^-$*tcpA*$^+$ and *ctxAB*$^-$*tcpA*$^-$) and CRISPR–Cas class 1 types I (subtypes I-E, I-F, I-C) and III (*ctxAB*$^-$*tcpA*$^-$) were identified. The detection of several types and subtypes of the CRISPR–Cas system in the genome of a number of *ctxAB*$^-$*tcpA* strains may indicate its repeated acquisition through horizontal transfer.

Keywords: *non-toxigenic strains of Vibrio cholerae, antiphage systems, CRISPR–Cas system class 1*

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Оригинальное исследование
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Анализ генов антифаговых систем нетоксигенных штаммов *Vibrio cholerae* O1 биовара Эль Тор

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Аннотация

Актуальность. Наличие и структура антифаговых систем, способствующих устойчивости холерных вибрионов к литическим фагам, у нетоксигенных штаммов *Vibrio cholerae* O1 биовара El Tor, выделенных на территории России и сопредельных стран, не изучена.

Цель — выявление и анализ антифаговых систем нетоксигенных штаммов *V. cholerae* O1 биовара El Tor.

Материалы и методы. В работе использовали 126 нетоксигенных (*ctxAB*$^{-}$*tcpA*$^{+}$ и *ctxAB*$^{-}$*tcpA*$^{-}$) штаммов *V. cholerae* O1 El Tor, изолированных с 1972 по 2018 г. Секвенирование ДНК проводили на платформе MGI DNBSEQ-G50. Для биоинформатического анализа применяли программы fastp v. 0.23, unicycler v. 0.4.7, Blast 2.16.0, MEGA X, CRISPRCastyper и CRISPRCasFinder.

Результаты. В геноме изученных штаммов не выявлены фагоиндуцируемые острова PLE, BREX и DISARM-системы. Установлено, что 80% *ctxAB*$^{-}$*tcpA*$^{+}$-штаммов содержат систему рестрикции–модификации I типа, у *ctxAB*$^{-}$*tcpA*$^{-}$-изолятов данная система не обнаружена. Гены CBASS-системы выявлены у единичных штаммов обеих групп. В геноме 35 (32%) изученных *ctxAB*$^{-}$*tcpA*$^{-}$-штаммов, выделенных в разных регионах РФ и сопредельных странах, установлено наличие CRISPR–Cas-системы 1-го класса типов I (подтипы I-E, I-F, I-C) и III (подтип III-B). Количество спейсеров в данной системе варьировало от 0 до 80, их последовательность была гомологична протоспейсерным участкам ДНК литических и умеренных фагов, транспозонов, плазмид *V. cholerae*, представителей рода *Vibrio* и неродственных бактерий. Наличие у ряда штаммов спейсеров, гомологичных генетическому материалу фага, циркулирующему на эндемичных территориях, может указывать на завозной характер данных штаммов.

Заключение. Выявлена гетерогенность изученных нетоксигенных штаммов *V. cholerae* O1 El Tor по наличию антифаговых систем, что расширяет сведения об их генетической организации. В их геноме выявлены системы рестрикции–модификации I типа (*ctxAB*$^{-}$*tcpA*$^{+}$), CBASS (*ctxAB*$^{-}$*tcpA*$^{+}$ и *ctxAB*$^{-}$*tcpA*$^{-}$) и CRISPR–Cas 1-го класса типов I (подтипы I-E, I-F, I-C) и III (*ctxAB*$^{-}$*tcpA*$^{-}$). Выявление нескольких типов и подтипов CRISPR–Cas-системы в геноме ряда *ctxAB*$^{-}$*tcpA*$^{-}$-штаммов может указывать на её неоднократное приобретение посредством горизонтального переноса.

Ключевые слова: *нетоксигенные штаммы Vibrio cholerae, антифаговые системы, CRISPR–Cas-система 1-го класса*

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

Every year, during monitoring studies of Russian water bodies for cholera, non-toxigenic strains of *Vibrio cholerae* O1 serogroup El Tor biovar are isolated, lacking the *ctxAB* genes (*ctxAB*$^-$) that encode the biosynthesis of cholera toxin (the main virulence factor of the cholera pathogen). Sometimes strains containing the *tcpA* gene *(ctxAB*$^-$*tcpA*$^+$), which is responsible for the production of the main subunit of toxin-coregulated pili of adhesion (colonization factor), are isolated. Both *ctxAB*$^-$*tcpA*$^-$ and *ctxAB*$^-$*tcpA*$^+$ strains do not cause cholera, but they could be the cause of acute intestinal infections. It has been established that non-toxigenic strains of *V. cholerae O1* El Tor can persist in environmental objects in Russia for a long time, forming clonal complexes, and are also imported from cholera-endemic countries [1–7]. To identify the mechanisms of long-term survival of non-toxigenic strains in the external environment, as well as their ability to cause acute intestinal infections, the molecular genetic characteristics of these strains are being actively studied, various kinds of typing are being performed, and phylogenetic analysis is being conducted. As a result, the presence of genes encoding additional virulence and persistence factors has been identified in the genomes of non-toxigenic strains, which can also act as toxic substances. These include loci that provide motility, are responsible for the biosynthesis of additional toxins, a thermolabile hemolysin, proteases, neuraminidase, type 6 and type 3 secretion system proteins, mannose-sensitive adhesion pili, as well as regulatory proteins that control the transcription of virulence and persistence genes [3, 5, 8]. However, the reasons for the long-term survival of non-toxigenic *V. cholerae* O1 El Tor strains in the external environment are not fully understood.

It should be noted that when in the water of open bodies of water, non-toxigenic *V. cholerae* O1 El Tor vibrios can be attacked by bacteriophages (phages), which are also present in this environment. It has been shown that cholera phages play an important role in the genetic diversity of *V. cholerae* strains [9]. The co-existence of *V. cholerae* and phages and the need for both to survive drive the evolution of both *V. cholerae* and phages [9, 10]. Bacteria acquire various mechanisms of resistance to phages through horizontal gene transfer, while phages, in turn, very quickly develop resistance to many bacterial defense systems. It has been found that genes encoding resistance to phages can make up more than 10% of a bacterial genome [11].

A significant number of antiphage systems located on mobile genetic elements have been identified in toxigenic *V. cholerae* O1 El Tor strains. This includes a gene cluster encoding a type I restriction-modification system (*vc1764–vc1769*) located on the VPI-2 pathogenicity island, whose action is based on the activity of two enzymes: a restriction endonuclease (*vc1765*) and a methyltransferase (*vc1769*) [12]. The VSP-I pathogenicity island contains the CBASS (cyclic-oligonucleotide-based antiphage signaling system) antiphage signaling system, which includes an operon of 4 genes: *dncV, capV, cap2, cap3* [13, 14]. The ICE SXT element contains BREX (Bacteriophage exclusion) and DISARM (Defence Islands System Associated with Restriction-Modification) systems [10, 15, 16].

A significant role in protection against the most common phage in the endemic area, ICP1, is played by Phage inducible chromosomal island-like elements (PLE), of which there are currently 10 types [17–19]. However, toxigenic El Tor vibrios lack the adaptive defense system CRISPR–Cas, which was identified in the genome of non-toxigenic *V. cholerae* O1 El Tor strains circulating in endemic areas [20]. This system includes clustered regularly interspaced short palindromic repeats (CRISPR), spacers (sequences of foreign origin), and *cas* genes encoding proteins with various functions [13]. Currently, the CRISPR–Cas system is classified into 2 classes, 6 types and 33 subtypes based on its mechanism of action, the structure of the CRISPR spacers, and the presence of *cas* genes.

In a Class 1 system (types I, III, IV), a multi-component complex consisting of several Cas proteins bound to crRNA interacts with the target. Class 2 systems (types II, V, and VI) contain only one protein (Cas9, Cas12, or Cas13) that performs all the functions of a multi-protein effector complex.

The classification into types is based on the structure of the effector complexes, with systems of the same type typically including a specific protein unique to that type of system. The types, in turn, are divided into subtypes, which differ in the structure of the CRISPR locus and, in certain cases, in the presence of Cas proteins. It is a fact that many Type I systems contain the *cas3* gene, which encodes the Cas3 helicase-nuclease. Through its helicase activity, it unwinds the foreign DNA double helix, and with the involvement of its nuclease domain, it fragments the foreign genetic material. In type III systems, the Cas10 protein exhibits nuclease activity. All types include the Cas1 and Cas2 proteins, which form a complex and are responsible for the adaptation stage, i.e., the insertion of a new spacer into the CRISPR array [20, 21].

Despite active research on *V. cholerae* antiphage systems, their presence in the genomes of non-toxigenic *V. cholerae* O1 El Tor strains isolated in Russia and neighboring countries has not been studied. Considering the above, the aim of the study was to identify and analyze the antiphage systems of non-toxigenic strains of *V. cholerae* O1 El Tor biovar.

Materials and methods

We studied the nucleotide sequences of the complete genomes of 126 non-toxigenic *V. cholerae* O1 El Tor strains isolated in Russia and neighboring countries from 1972 to 2018. The nucleotide sequences of 30

strains (12 *ctxA⁻tcpA⁺* and 18 *ctxA⁻tcpA⁻*) were obtained from the NCBI GenBank database, and 96 strains (3 *ctxA⁻tcpA⁺* and 93 *ctxA⁻tcpA⁻*) were sequenced in this study. For sequencing, the strains were obtained from the State Collection of Pathogenic Bacteria of the Russian Anti-Plague Institute "Microbe" of Rospotrebnadzor, where they were stored in a lyophilized state.

Genomic DNA was prepared according to the manufacturer's protocol from a sodium merthiolate-treated bacterial suspension using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences).

Sequencing was performed on the MGI DNBSEQ-G50 platform (MGI). Libraries were prepared according to a standard protocol using the DNBSEQ-G50RS (FCLPE150) and MGI EasyFast-PCR-FREEFS Library PrepSet (MGI) kits. Quality control of the obtained reads was performed using the fastpv.0.23 program, and contigs were assembled using unicyclerv.0.4.7.

The Blast 2.16.0 algorithm (http://blast.ncbi) and the MEGA X program (or BioEditV. 7.0.9.0) were used for bioinformatics analysis. CRISPR–Cas systems and spacers were identified using the CRISPRCastyper (https://github.com/Russel88/CRISPRCasTyper) and CRISPRCasFinder (https://github.com/dcouvin/CRISPRCasFinder) programs.

Results

When studying the nucleotide sequences of complete genomes of non-toxigenic strains, phage-inducible PLE islands were not found in the genomes of either *ctxAB⁻tcpA⁻* or *ctxAB⁻tcpA⁺* strains. The BREX and DISARM systems were also not detected, as these strains lack the ICE SXT elements.

Analysis of type I restriction-modification system genes (*vc1765*, *vc1769*) among 15 *ctxAB⁻tcpA⁺* strains revealed 12 isolates that matched the toxigenic *V. cholerae* N16961 O1 El Tor reference strain for these genes (**Table**). In *ctxAB⁻tcpA⁻* strains, type I restriction-modification system genes were not detected.

The presence of CBASS system genes (*dncV*, *capV*) was established in 4 strains. In the *ctxAB⁻tcpA⁺* strain of *V. cholerae* 56, the gene data structure did not differ from that of the *V. cholerae* N16961 O1 El Tor reference strain. In *ctxAB⁻tcpA⁻ V. cholerae* strains P-18748, 102, and M-1457, the *capV* gene was intact, and identical non-synonymous point mutations were identified in the *dncV* gene, leading to amino acid sub-

Characteristics of certain non-toxigenic *V. cholerae* O1 El Tor strains and the structure of their anti-phage genes

Strain	Location, year	Source of isolation	Gene structure		Type of CRISPR–Cas systems	Amount of spacers
			vc1765/ vc1769	vc0179 (dncV)/ vc0178 (capV)		
Non-toxigenic *ctxAB⁻tcpA⁺*-strains						
M1395[LQBY01]	Russia, Astrakhan, 1981	Environment	–	–	–	–
56[MWRD01]	Ukraine, Mariupol, 1995	Infected patient	int	int	–	–
866[MWRF01]	Ukraine, Yalta, 1996	Environment	int	–	–	–
85[NEDU01]	Ukraine, Berdyansk, 1999	Infected patient	int	–	–	–
P18778[NIFI01]	Russia, Rostov-on-Don, 2005	Infected patient	–	–	–	–
M1434*, M1436*	Russia, Kalmykia, 2006	Environment	int	–	–	–
M1501[LRAE01]	Russia, Kalmykia, 2011	Infected patient	–	–	–	–
M-1504[VTLN01]	Russia, Kalmykia, 2011, 2012	Environment	int	–	–	–
M-1518[LQZR01]	Russia, Kalmykia, 2011, 2012	Environment	int	–	–	–
M-1524[LQZS01], M1528*	Russia, Kalmykia, 2013	Environment	int	–	–	–
2613[PYCA01], 2687[PYCB01]	Russia, Kalmykia, 2015	Environment	int	–	–	–
124[PYCD01]	Russia, Kalmykia, 2017	Environment	int	–	–	–
Non-toxigenic *ctxAB⁻tcpA⁻*-strain						
M-988[LQBX01]	Turkmenistan, 1972	Environment	–	–	I-F mini	Undetermined
M-658*	Russia, Ufa, 1976	Environment	–	–	I-F mini	1
M-659*	Russia, Salavat, 1976	Environment	–	–	I-E	56
M-1114*	Russia, Saransk, 1977	Infected patient	–	–	I-E I-F mini	13 0
M-1115*	Russia, Saransk, 1977	Infected patient	–	–	I-E I-F mini	13 1
M-1394*	Russia, Kaspiysk, 1979	Environment	–	–	I-E	44

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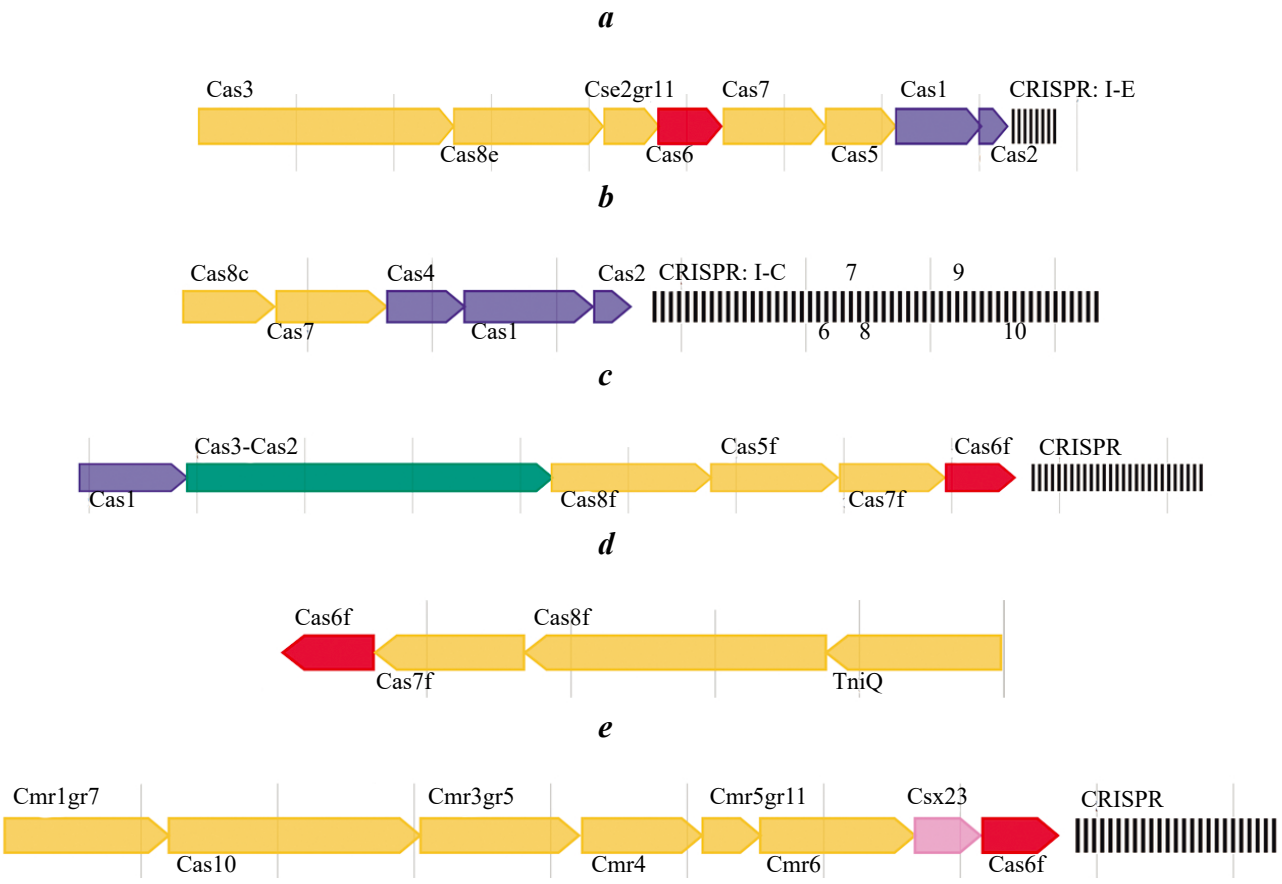
Continuation of the Table

Strain	Location, year	Source of isolation	Gene structure		Type of CRISPR–Cas systems	Amount of spacers
			vc1765/ vc1769	vc0179 (dncV)/ vc0178 (capV)		
M-1222*	Russia, Astrakhan, 1985	Environment	–	–	I-F mini	10
M-1320*	Russia, Saratov, 1998	Environment	–	–	III-B	24
617[NCTY01]	Ukraine, 1999	Infected patient	–	–	I-F** I-E III-B	26 80 25
M1337[NEEB01]	Russia, Astrakhan, 2000	Infected patient	–	–	I-E**	1
M-1388*	Russia, Saratov, 2001	Environment	–	–	I-E I-F mini	13 1
M-1389*	Russia, Saratov, 2001	Environment	–	–	I-E I-F mini	13 1
M-1411*	Russia, Kalmykia, 2002	Infected patient	–	–	I-F mini	1
M-1413*	Russia, Kalmykia, 2002	Environment	–	–	I-C I-F mini	40 2
M-1426*	Russia, Perm Krai, 2003	Environment	–	–	I-F mini	0
M-1428*	Russia, Astrakhan, 2003	Environment	–	–	I-E	44
M-1431*	Russia, Kalmykia, 2005	Environment	–	–	I-E	60
P-18748[NIFH01]	Russia, Sochi, 2004	Infected patient	–	A1003G (S335G) /int	–	–
102[NDXO01]	Ukraine, 2006	Infected patient	–	A1003G (S335G) /int	I-F**	49
M-1441*	Russia, Kalmykia, 2007	Environment	–	–	I-E	48
M-1443*	Russia, Kalmykia, 2007	Environment	–	–	I-E	58
M-1444*	Russia, Kalmykia, 2007	Environment	–	–	I-E	76
M-1447*	Russia, Kalmykia, 2009	Environment	–	–	I-F	15
M-1450*	Russia, Kalmykia, 2009	Environment	–	–	I-F mini	1
M1457[VTLH01]	Russia, Kalmykia, 2009	Environment	–	A1003G (S335G) /int	I-F	2
M-1460*	Russia, Tatarstan, 2010	Environment	–	–	I-E I-F mini	14 1
2403[NEDV01]	Ukraine, 2011	Infected patient	–	–	I-E I-F mini	12 0
M-1486*	Russia, Tatarstan, 2011	Environment	–	–	I-E I-F mini	29 1
M-1487*	Russia, Tatarstan, 2011	Environment	–	–	I-E I-F mini	15 0
M1516[VTZY01]	Russia, Kalmykia, 2012	Environment	–	–	I-F I-C	58 54
M1517[VTZZ01]	Russia, Kalmykia, 2012	Environment	–	–	I-E	23
M-1525*	Russia, Kalmykia, 2012	Environment	–	–	I-F mini	10
M1526[VUAA01]	Russia, Kalmykia, 2012	Environment	–	–	I-F I-C	58 54
29[VUAB01]	Russia, Kalmykia, 2013	Environment	–	–	I-E	7
M-1543*	Russia, Kalmykia, 2017	Environment	–	–	I-E I-F mini	29 1
136[VTLK01]	Russia, Kalmykia, 2018	Environment	–	–	I-E I-F mini	8 3

Note. The strain superscript indicates the NCBI GenBank accession code. *Strains sequenced in this study. "–" — gene(s) not detected; int — the gene structure corresponds to the *V. cholerae* N16961 O1 El Tor reference strain. **The presence of the CRISPR–Cas system was previously established [20].



Structure of CRISPR–Cas systems in the studied *ctxAB*⁻*tcpA*⁻ *V. cholerae* O1 El Tor strains.

a — canonical subtype I-E, present in *V. cholerae* strain 29; *b* — subtype I-C of *V. cholerae* strain M1526; *c* — subtype I-F of *V. cholerae* strain 617; *d* — subtype I-F mini of *V. cholerae* strain M1426; *e* — subtype III-B of *V. cholerae* strain M1320. The spacers (CRISPR) are marked by black vertical lines on the right.

stitutions whose impact on the functional role of the DncV protein is unknown. In the genomes of other strains, the system mentioned was absent (Table).

Next, the presence of the CRISPR–Cas system was investigated. This system was not found in the genomes of *ctxAB*⁻*tcpA*⁺ strains, while among the *ctxAB*⁻*tcpA*⁻ strains, 35 isolates were identified that have a Class 1 Type 2 CRISPR–Cas system — Types I and III. Type I was represented by three subtypes: I-E, I-F, I-C, while Type III was represented by one (III-B). The largest number of strains (20 isolates) were found to have a subtype I-E CRISPR–Cas system, of which 10 isolates had only this subtype, while the others had additional systems. Thus, strain *V. cholerae* 617, in addition to I-E, had 2 more systems (Table). It should be noted that the I-E system structure was canonical in all strains and consisted of 8 *cas* genes (*cas3, cas8e, cse2, cas6, cas7, cas5, cas1, cas2*) (**Figure,** *a*).

Three strains — *V. cholerae* M1413, M1516, M1526 — belonged to subtype I-C (Table, Figure, *b*). Five strains (*V. cholerae* 617, 102, M1457, M1516, M1526) contained a complete subtype I-F CRISPR–Cas system with 6 *cas* genes (*cas1, cas3, cas8f, cas5, cas7, cas6f*: Table, Figure, *c*). A truncated I-F system (*cas6f, cas7f, cas8f*), consisting of 3 *cas* genes, which we have designated I-F mini (Figure, *d*), was identified in a number of strains. It should be noted that this system lacks the *cas3* gene. Two strains, *V. cholerae* M1320 and 617, were type III, subtype III-B (*cmr1, cas10, cmr3, cmr4, cmr5, cmr6, csx23, cas6f*; Table, Figure, *e*)

Non-toxigenic *ctxA*⁻*tcpA*⁻ strains with the CRISPR–Cas system are quite widespread in Russia. These strains were isolated in different years in the Republics of Tatarstan, Dagestan, Bashkortostan, Mordovia, Kalmykia, as well as in the Astrakhan, Saratov regions and the Perm Krai (Table).

The next stage of the study was dedicated to identifying spacers. They were present in almost all systems, with their number varying from 1 to 80, and the largest number of spacers was found in system I-E. The exception was 4 strains containing the I-F mini system, in which spacers were absent (Table). Due to the poor quality of the whole-genome nucleotide sequence presented in GenBank, it was not possible to reliably identify the spacers in strain M-988. The identified spacers were homologous to the protospacer sequences of a large number of lytic and temperate phages, as well as

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plasmids and the *V. cholerae* transposon (phages: O395, VPUSM 8, K139, K491, K571, K575, VcP032, Kappa, Rostov 7, X29, phi 2, JSF1, JSF2, JSF4, JSF5, JSF6, JSF13, JSF14, JSF17, VMJ710, Rostov M3, CP-T1, 24, vB_VchM-138, vB_VchM_VP-3213, Ch457, E8498, fs1, fs2, Vb_VaM_Valp1, ICP1, VRU, VP24-2_Ke, VMJ710, VcP032, VEJphi, VSK, VSKK, ND1-fs1, KSF-1phi, VGJphi, 1.178). O.J. L 286. 45. E12, 1.028. O.J. L 286. 45. B6, 1.159. O.J. L 261. 46. F12, Martha 12B12, Jenny 12G5, vB_Vipa26, vB_Vipa10, vB_Vipa4291, vB_Vipa71, vB_VpS_PG07, Zoerhiza.4_15, 13VV501A, 6E35-1b, D481, D483, D485, D491s, D527, VaK; plasmids: HDW18, pSA7G1, pSA7G2; transposon Tn7005). Furthermore, spacers homologous to the nucleotide sequences of phages and plasmids from *Vibrio* genus (*V. alginolyticus, V. vulnificus, V. parahaemolyticus, V. fluvialis, V. furnissii, V. nigripulchritudo, V. metschnikovii*), as well as from unrelated bacterial species (*Klebsiella* spp., *Escherichia* spp., *Salmonella* spp., *Shigella*, *Shewanella algae*, *Xanthomonas*, *Stenotrophomonas*) were identified. It is worth noting the presence of spacers in certain strains (*V. cholerae* 102, M1428, M1431, M1443, M1444, M1457, M1460, M1486) that are identical to DNA sequences of phage ICP1 (the most common bacteriophage in endemic areas), isolated in different years in the Democratic Republic of Congo and Bangladesh.

Thus, the following antiphage systems were identified in the genomes of the studied non-toxigenic *V. cholerae* O1 El Tor strains: type I restriction-modification (*ctxAB⁻tcpA⁺*), CBASS (*ctxAB⁻tcpA⁺* and *ctxAB⁻tcpA⁻*) and class 1 CRISPR–Cas (*ctxAB⁻tcpA⁻*).

Discussion

Given that non-toxigenic *V. cholerae* O1 El Tor strains circulate in open water bodies, which are also habitats for various cholera phages, it was expected that a large number of antiphage systems would be found in their genomes. However, these strains lack the PLE, BREX, and DISARM antiphage islands of the system. Type I restriction-modification systems, which were found in the genome of *ctxAB⁻tcpA⁺* strains, were not detected in *ctxAB⁻tcpA⁻* strains. CBASS system genes are present in a few strains from both groups. Meanwhile, 35 *ctxAB⁻tcpA⁻* strains studied, isolated from aquatic environments in various regions of Russia and neighboring countries, contain a Class 1 CRISPR–Cas system of types I and III, which is absent in *ctxAB⁻tcpA⁺* isolates. However, subtypes I-C and III-B were detected in only a few strains, while 57% of the strains have the canonical type of the type I-E system. Our data confirm previously obtained information from the analysis of strains circulating in the endemic area regarding the widespread distribution of the I-E system among non-toxigenic *V. cholerae* strains [20]. A number of authors suggest that the stable maintenance of the I-E system structure is due to its location on the GI-24 genomic island, and its transfer to other strains occurs only as part of this mobile genetic element [20, 22]. Certain strains also included a complete I-F system, which is part of the newly discovered VPI-6 (Vibrio Pathogenicity Island), capable, similar to GI-24, of being entirely excised from the chromosome and transferred to other cells [23]. At the same time, most strains had the I-F mini system, which has the *tniQ* gene responsible for transposase production located next to the *cas* gene loci. According to literature data, such systems are associated with the Tn7 transposon [20]. Given the absence of the *cas3* gene encoding a helicase-nuclease in this system, it can be assumed that I-F mini is non-functional.

Since new spacers are primarily inserted into the 5' regions of the system, CRISPR represents a chronological record of the bacterium's interaction with mobile genetic elements. In this regard, the presence of spacers homologous to the genetic material of phage ICP1 circulating in endemic areas may indicate the imported nature of these strains. It is also evident that by protecting *V. cholerae* from predation by cholera phages, as well as phages of other bacteria, the CRISPR–Cas system increases the survival of non-toxigenic strains in the external environment. Perhaps its presence is one of the mechanisms for the long-term circulation of non-toxigenic strains in open water bodies.

Thus, the studied non-toxigenic *ctxAB⁻tcpA⁺* and *ctxAB⁻tcpA⁻ V. cholerae* O1 El Tor strains isolated in Russia and neighboring countries lack a number of mobile genetic elements with anti-phage loci (PLE islands, ICE SXT elements with BREX and DISARM systems). At the same time, a type I restriction-modification system was identified in the genome of *ctxAB⁻tcpA⁺* strains, and a CBASS (*ctxAB⁻tcpA⁺* and *ctxAB⁻tcpA⁻*) and a class 1 CRISPR–Cas system (*ctxAB⁻tcpA⁻*) were identified in one strain.

Conclusion

The research conducted has established the heterogeneity of the studied non-toxigenic *V. cholerae* O1 El Tor strains circulating in Russia and neighboring countries in terms of the presence of antiphage systems located on mobile genetic elements, which expands our knowledge of their genetic organization. It was found that 80% of the studied *ctxAB⁻tcpA⁺* strains contain a type I restriction–modification system, which was not detected in *ctxAB⁻tcpA⁻* strains. Single strains (1 *ctxAB⁻tcpA⁺* and 3 *ctxAB⁻tcpA⁻*) have a CBASS system, which is intact in the *ctxAB⁻tcpA⁺* isolate and corresponds to the toxigenic reference strain *V. cholerae* N16961 O1 El Tor. A class 1 CRISPR–Cas system of type I (subtypes I-E, I-F, I-F mini, I-C) and type III (subtype III-B), which is absent in *ctxAB⁻tcpA⁺* strains, was identified in the genome of 32% of the ctxAB-tcpA- strains studied. The most common (57%) is the canonical system of subtype I-E. The presence of multiple types and

subtypes of the CRISPR–Cas system in the genome of several strains may indicate its repeated acquisition by these isolates through horizontal transfer. Analysis of spacers in the CRISPR cassette allows for the identification of non-toxigenic *V. cholerae* O1 El Tor strains imported from cholera-endemic areas.

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Determinants of resistance to levofloxacin and metronidazole in Russian clinical isolates of *Helicobacter pylori* based on whole-genome sequencing data

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Abstract

Introduction. Helicobacter pylori infection, however, data on the mechanisms of metronidazole (MTZ) and levofloxacin (LVX) resistance in Russia remain scarce. and levofloxacin (LVX) in Russia.

The aim of the study is to identify the determinants of resistance in clinical isolates of *H. pylori* to MTZ and LVX using whole-genome sequencing data.

Materials and methods. A retrospective analysis of 43 *H. pylori* isolates obtained from adult patients (2014–2022) was conducted. Susceptibility to antibiotics was determined using the bacteriological disk diffusion method. Whole-genome sequencing of 43 *H. pylori* strains was performed using a DNBSEQ-G50 sequencer.

Results. The evaluation of the phenotypic drug susceptibility test results showed that 11 isolates were susceptible to MTZ (MTZ-S), 31 were susceptible to LVX (LVX-S), while 32 isolates were resistant to MTZ (MTZ-R), and 12 were resistant to LVX (LVX-R). To identify the association between phenotypic and genotypic resistance, an analysis of nucleotide substitutions in the *gyrA, gyrB, rdxA, frxA, fdxB* and *fur* genes was conducted. Of all the mutations identified in the *gyrA* and *gyrB* genes, only *D91/GNY* in the *gyrA* gene was associated with phenotypic resistance to LVX and was found in 4/12 (33.3%) of the isolates ($p < 0.05$). The combined mutation *D91G/N/Y+N87K* in the *gyrA* gene was detected in 6/12 (50.0%) of LVX-R isolates ($p < 0.001$). Point mutations in the *rdxA* gene were detected in 21.9% (7/32) of MTZ-R isolates, leading to a frameshift or premature termination of protein synthesis. None of the mutations in the *frxA*, *fur* and *fdxB* genes were associated with *H. pylori* resistance to MTZ.

Conclusion. Based on the results of whole-genome sequencing of Russian clinical isolates of *H. pylori*, the detection of the combined mutation *D91G/N/Y+N87K* in the *gyrA* gene can serve as a predictor of the phenotypic resistance of *H. pylori* to levofloxacin.

Keywords: *Helicobacter pylori, whole-genome sequencing, antibiotic resistance, resistance determinants, metronidazole, levofloxacin*

Ethics approval. The study was conducted in strict compliance with confidentiality standards: all patient data was made anonymous and encrypted. Informed consent was obtained from each study participant. The study protocol was approved by the Independent Local Ethics Committee of the Pasteur Research Institute of Epidemiology and Microbiology (protocol No. 50/04–2019 dated June 22, 2020).

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Оригинальное исследование
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Детерминанты резистентности к левофлоксацину и метронидазолу российских клинических изолятов *Helicobacter pylori* по результатам полногеномного секвенирования

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Аннотация

Введение. Устойчивость к антибактериальным препаратам является одной из ключевых проблем в лечении *Helicobacter pylori*-инфекции, однако в России практически отсутствуют данные о механизмах резистентности к метронидазолу (MTZ) и левофлоксацину (LVX).

Цель работы — выявление детерминант резистентности у клинических изолятов *H. pylori* к MTZ и LVX с использованием данных полногеномного секвенирования.

Материалы и методы. Проведён ретроспективный анализ 43 изолятов *H. pylori*, выделенных от взрослых пациентов (2014–2022 гг.). Чувствительность к антибактериальным препаратам определяли бактериологическим диско-диффузионным методом. Полногеномное секвенирование 43 штаммов *H. pylori* проводили с использованием секвенатора «DNBSEQ-G50».

Результаты. Оценка результатов теста фенотипической лекарственной чувствительности показала, что 11 изолятов являлись чувствительными к MTZ (MTZ-S), 31 — чувствительными к LVX (LVX-S), тогда как 32 изолята проявляли устойчивость к MTZ (MTZ-R), 12 — к LVX (LVX-R). Для выявления ассоциации между фенотипической и генотипической устойчивостью проведён анализ нуклеотидных замен в генах *gyrA*, *gyrB*, *rdxA*, *frxA*, *fdxB*, *fur*. Из всех мутаций, выявленных в генах *gyrA* и *gyrB*, только *D91/GNY* в гене *gyrA* была ассоциирована с фенотипической устойчивостью к LVX и обнаружена у 4/12 (33,3%) изолятов ($p < 0,05$). Комбинированная мутация *D91G/N/Y+N87K* в гене *gyrA* выявлена у 6/12 (50,0%) LVX-R-изолятов ($p < 0,001$). У 21,9% (7/32) MTZ-R-изолятов в гене *rdxA* выявлены точечные мутации, приводящие к сдвигу рамки считывания или преждевременной терминации синтеза белка. Ни одна из мутаций в генах *frxA*, *fur* и *fdxB* не была ассоциирована с устойчивостью *H. pylori* к MTZ.

Выводы. По результатам полногеномного секвенирования российских клинических изолятов *H. pylori* детекция комбинированной мутации *D91G/N/Y+N87K* в гене *gyrA* может служить предиктором фенотипической устойчивости *H. pylori* к LVX.

Ключевые слова: *Helicobacter pylori, полногеномное секвенирование, устойчивость к антибактериальным препаратам, детерминанты резистентности, метронидазол, левофлоксацин*

Этическое утверждение. Исследование проведено с соблюдением строгих норм конфиденциальности: все данные пациентов деперсонифицированы и зашифрованы. От каждого участника исследования получено информированное согласие. Протокол исследования одобрен независимым локальным этическим комитетом НИИ эпидемиологии и микробиологии им. Пастера (протокол № 50/04–2019 от 22.06.2020).

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

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Introduction

Helicobacter pylori infection remains one of the most common chronic bacterial infections worldwide and is considered a major risk factor for the development of gastric cancer (approximately 90% of cases) [1]. According to the key principles of the Maastricht VI/Florence Consensus, which underpin national clinical guidelines, *H. pylori* infection (regardless of symptoms or complications) invariably causes gastritis, for which the only treatment is eradication therapy. Furthermore, the first principle of the consensus recommends conducting drug susceptibility testing prior to prescribing first-line therapy to ensure the rational use of antibiotics [2, 3].

H. pylori resistance to antibiotics is currently recognized as one of the most serious issues. Eradication therapy for *H. pylori* typically uses a combination of 2–3 antibiotics (such as amoxicillin, clarithromycin, metronidazole (MTZ), tetracycline, levofloxacin (LVX), or rifabutin), a proton pump inhibitor, and, in some regimens, a bismuth [2, 3]. However, the efficacy of *H. pylori* treatment has been steadily declining in recent years, in parallel with the growth of antibiotic resistance [4, 5]. Furthermore, the widespread use of antibiotics and low patient compliance not only foster resistance in *H. pylori* but also exert selective pressure on the broader gastrointestinal microbiome. This promotes the selection of resistance genes, enriches the resistome, and facilitates the spread of these genes among bacterial communities [6, 7].

The genetic mechanisms underlying the development of antibiotic resistance (AR) in *H. pylori* are not fully understood. It is generally believed that *H. pylori* resistance to antibiotics is due to *de novo* mutations in chromosomal DNA, which either alter the target of the antibiotic or prevent its activation within cells [6]. Nevertheless, a significant proportion of *H. pylori* strains resistant to antibiotics lack known genetic determinants of resistance. This indicates the complex and multifactorial nature of resistance mechanisms, which go beyond mutational activity and/or the activation of individual genes in response to antibiotic use. Potential mechanisms of *H. pylori* resistance include:

- increased expression of efflux system genes;
- synergistic interactions involving mutations, horizontal gene transfer, and activation of protective systems;
- cellular adaptation associated with the formation of biofilms and antibiotic-resistant coccoid forms;

Compensatory mutations that mitigate the fitness cost of resistance through epistatic interactions with resistance determinants;heteroresistance — a phenomenon where subpopulations of cells with different susceptibility to antibiotics are present simultaneously within a bacterial population.

All the mechanisms listed above not only contribute to the formation of *H. pylori* antibiotic resistance but also accelerate the development of multidrug resistance, which significantly complicates the eradication process and highlights the necessity for a thorough evaluation and improvement of therapeutic approaches [6–8].

The mechanism of action of MTZ, a first-line drug for *H. pylori* eradication therapy, involves the reduction of the nitro group under anaerobic conditions, leading to the formation of cytotoxic nitroanions and free radicals that damage DNA and disrupt the functioning of bacterial cells. *H. pylori* resistance to MTZ is due to a complex interplay of genetic and biochemical processes that are not yet fully understood. The main mechanism of resistance to MTZ is mediated by the inactivation of the reductase enzyme genes *rdxA* (which encodes an oxygen-insensitive nitroreductase) and *frxA* (which encodes flavin oxidoreductase), which reduces the ability of MTZ to be reduced to its active forms (NO_2^- and NO_2^{2-}) and, consequently, diminishes the drug's antimicrobial effect [9, 10]. Mutations in the *fur* gene, which regulates iron uptake, and *fdxB*, which encodes ferredoxin, are also thought to contribute to the formation of AR [7, 8, 10]. Increased levels of antioxidant enzymes and the efflux system, as well as mutational activity in genes responsible for repairing damaged DNA, act as additional factors contributing to the development of *H. pylori* resistance to MTZ [6].

Another antibiotic used as a reserve treatment for *Helicobacter pylori* infection, LVX, exerts its antibacterial effect by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV — key enzymes involved in DNA replication and recombination processes. The most common mechanism of *H. pylori* resistance to fluoroquinolones is due to point mutations in the quinolone resistance-determining regions (QRDR), particularly in codons 86, 87, 88, 91, 97 of the *gyrA* gene and codons 481, 484 and 463 of the *gyrB* gene [6, 7, 10]. However, the role of some of these mutations in the development of *H. pylori* resistance to LVX has not been proven.

The systematic and reliable data on the resistance rates of *H. pylori* to LVX and MTZ in Russia are scarce. Additionally, the genetic determinants of resistance to these antibiotics remain largely uncharacterized, impeding the development of effective molecular diagnostic tools for monitoring resistance in clinical practice. **The aim** of our study was to identify the determinants of resistance in *H. pylori* clinical isolates to MTZ and LVX using whole-genome sequencing data.

Materials and methods

A retrospective analysis was conducted on 43 clinical isolates of *H. pylori* obtained from adult patients with gastrointestinal diseases (2014–2022) at the Pasteur Research Institute of Epidemiology and Microbio-

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logy in St. Petersburg. The average age of the patients was 44.0 ± 4.5 years (range 22–70 years).

Biopsies from the gastric antrum and body, were placed in thioglycolate medium, homogenized and subsequently cultured on selective medium based on Columbia agar supplemented with 7% defibrinated horse blood and 1% IsoVitalex solution at 37°C under microaerophilic conditions (oxygen content ~5%) using GasPak 100 gas-generating pouches (BBL CampyPak Plus Microaerophilic System envelopes with Palladium Catalyst, BD Biosciences). *H. pylori* was identified using a set of biochemical tests (urease, catalase and oxidase) and a reagent kit for detecting *H. pylori* DNA by polymerase chain reaction (DNA-Technology).

To perform the antibiotic susceptibility test, the *H. pylori* bacterial culture was suspended to a density of 2 on the McFarland scale (~ 6×10^8 CFU/mL), 0.1 mL was applied to the surface of a Petri dish containing Müller–Hinton agar supplemented with 5% defibrinated horse blood, and evenly distributed across the surface with a spatula. The susceptibility of *H. pylori* isolates to MTZ and LVX was determined using the disk diffusion method: immediately after inoculation onto the agar surface, disks containing MTZ (5 µg/disk) and LVX (5 mcg/disk) were aseptically applied and incubated under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 72 hours. After incubation was complete, the diameter of the zones of complete growth inhibition around the antibiotic disk was measured in millimeters. The interpretation of the disk diffusion method results was based on the threshold values presented in the publication by Z. Zhong et al.: *H. pylori* strains were considered resistant to MTZ (MTZ-R) when the inhibition zone diameter was ≤ 16 mm, and susceptible (MTZ-S) when the diameter was ≥ 17 mm; resistant to LVX (LVX-R) when the inhibition zone diameter was ≤ 17 mm, and susceptible (LVX-S) when the diameter was ≥ 18 mm [11].

Total DNA from pure cultures of *H. pylori* was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH) according to the manufacturer's instructions. The DNA concentration of each sample was quantified using a Qubit 4.0 fluorometer. Whole-genome sequencing was performed on a DNBSEQ-G50 sequencer (MGI Tech Co. Ltd.).

The quality assessment of paired-end libraries, adapter removal, and low-quality sequence trimming (Q-score < 20) were performed using the FastQC v. 0.12.1 and Trim Galore! v. 0.6.7 programs. Bacterial genomes were assembled *de novo* using the genomic assembler SPAdes v. 3.13.1, and the results were evaluated using QUAST v. 5.2.0 [12, 13]. The obtained genomic sequences were aligned to the *H. pylori* 26695 reference strain (GenBank acc. no. AE000511.1). The Snippy v.4.6.0 program was used to assess genetic variations between isolates and identify nucleotide substitutions[1]. Aligned nucleotide sequences were visually analyzed using UGENE v. 38.1 [14]. All genome assemblies of clinical isolates of *H. pylori* were deposited in the NCBI GenBank database under registration number PRJNA1011037[2].

Statistical analysis was performed using the R programming language v. 4.3.2. The agreement between phenotypic and genotypic resistance profiles was evaluated using the χ^2 test and Fisher's exact test. Differences between groups were considered significant at $p < 0.05$.

Results

Phenotypic drug susceptibility testing of 43 clinical *H. pylori* isolates showed that 12 isolates were resistant to LVX (LVX-R), 32 to MTZ (MTZ-R); at the same time, 31 isolates were susceptible to LVX (LVX-S), and 11 to MTZ (MTZ-S). Among the resistant isolates, 9 were resistant to both antibiotics simultaneously (Group ***A***), while among the susceptible isolates, 8 were susceptible to both LVX and MTZ (Group ***B***: Fig. 1).

To identify the determinants of resistance to LVX and MTZ and their association with phenotypic resistance, all 43 isolates underwent whole-genome sequencing, followed by an analysis of nucleotide substitutions in the *gyrA, gyrB, rdxA, frxA, fdxB* and *fur* genes.

Of all the mutations in the *gyrA* gene, only D91N/Y/G was associated with phenotypic drug resistance and was detected in 33.3% (4/12) of LVX-R isolates ($p < 0.05$) (**Table**). The missense mutation *N87K* in the *gyrA* gene was detected in 16.7% (2/12) of LVX-R isolates and was not found in combination with *D91N/Y/G* and *A88P* mutations, while the *A88P* mutation was identified in only 1 (8.3%) resistant strain in combination with the *D91N* mutation. Given the absence of these mutations in LVX-S isolates, the combined *D91N/Y/G+N87K* mutation is significantly associated with resistance in clinical isolates and was detected in 50.0%

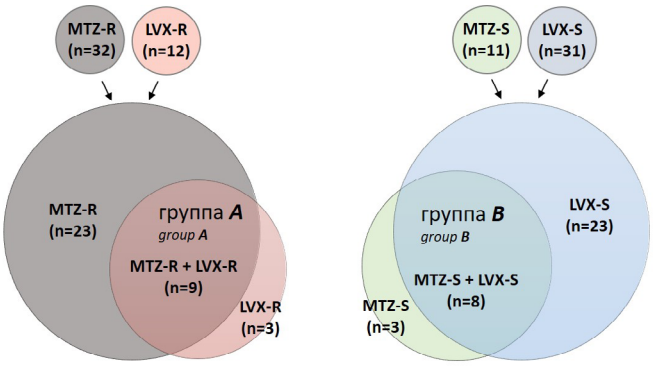

Fig. 1. Venn diagram showing the combinations of phenotypic drug susceptibility statuses of clinical isolates of *H. pylori* to MTZ and LVX

[1] URL: https://github.com/tseemann/snippy
[2] URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1011037

(6/12) of LVX-R isolates ($p < 0.001$). It should be noted that the *D91N/Y/G*, *N87K* and *A88P* mutations in the *gyrA* gene were found exclusively in group A isolates and were absent in monoresistant isolates. All mutations in the *gyrB* gene were present in *H. pylori* isolates regardless of their phenotypic resistance ($p > 0.05$). Mutations at positions 86 and 97 of the *gyrA* gene and 463 of the *gyrB* gene, which are presumably associated with *H. pylori* resistance to LVX, were not found in our sample.

In total, 56 point mutations in the *rdxA* gene were identified. Among these, 4 (*H97fs, S43fs, I182fs, M120fs*) were frameshift mutations, 2 (*Q50*stop*, *W52*stop*) were nonsense mutations leading to premature termination of the reading frame, and *Ter211R_ext** mutation resulted in the loss of stop codon, and fusion of *rdxA* gene with the adjacent *HP_0953* gene. Two mutations (*S108A/P, L62V*) identified for the first time in MTZ-R isolates, showed no association with phenotypic resistance ($p > 0.05$). The *R131K*, *T31E* and *D59N* mutations were found in *H. pylori* isolates from both phenotypic groups and were not associated with phenotypic resistance to MTZ.

We identified 7 frameshift mutations in the *frxA* gene, 4 of which (*Y19fs, R23fs, I44fs, A70fs*) were detected only in MTZ-R isolates, while 3 (*K18fs, R106fs,*

Mutations in LVX and MTZ resistance genes in *H. pylori* clinical isolates of compared to the *H. pylori* 26695 reference genome, *n* (%)

Gene (locus)	Amino acid substitution	LVX-R (*n* = 12)	LVX-S (*n* = 31)	MTZ-R (*n* = 32)	MTZ-S (*n* = 11)	*p*
gyrA (HP_0701)	*D91N/Y/G*	4 (33.3)	0			0.0040
	N87K	2 (16.7)	0			0.0730
	A88P	1 (8.3)	0			0.2790
gyrB (HP_0501)	*D481E*	3 (25.0)	8 (25.8)			~ 1.0000
	R484K	3 (25.0)	8 (25.8)			~ 1.0000
rdxA (HP_0954)	*S108A/P*			7 (21.9)	0	0.1628
	R16C			7 (21.9)	0	0.1628
	L62V			6 (18.7)	0	0.3122
	Ter211R_ext stop lost & splice region*			1 (3.1)	0	~ 1.0000
	*Q50*stop*			1 (3.1)	0	~ 1.0000
	*W52*stop*			1 (3.1)	0	~ 1.0000
	H97fs			1 (3.1)	0	~ 1.0000
	S43fs			1 (3.1)	0	~ 1.0000
	I182fs			1 (3.1)	0	~ 1.0000
	M120fs			1 (3.1)	0	~ 1.0000
	R131K			12 (37.5)	1 (9.1)	0.1290
	T31E			13 (40.6)	5 (45.4)	0.7794
	D59N			30 (93.7)	9 (81.8)	0.2665
frxA (HP_0642)	*K18fs*			17 (53.1)	5 (45.4)	0.6606
	Y19fs			1 (3.1)	0	~ 1.0000
	*Q27*stop*			1 (3.1)	0	~ 1.0000
	R23fs			1 (3.1)	0	~ 1.0000
	I44fs			1 (3.1)	0	~ 1.0000
	A70fs			1 (3.1)	0	~ 1.0000
	R106fs			1 (3.1)	1 (9.1)	0.4507
	*W137*stop*			1 (3.1)	0	~ 1.0000
	*Q141*stop*			1 (3.1)	0	~ 1.0000
	V215fs			10 (31.2)	6 (54.5)	0.1679
fdxB (HP_1508)	*N424fs*			1 (3.1)	1 (9.1)	0.4507
	K426fs			1 (3.1)	1 (9.1)	0.4507
fur (HP_1027)	*C150Y*			3 (9.4)	2 (18.2)	0.5890
	N118Q			7 (21.9)	3 (27.3)	0.6982

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V215fs) were found in isolates from both phenotypic groups. At the same time, 3 nonsense mutations leading to premature protein termination were found exclusively in MTZ-R strains (Table). In the *fdxB* gene, 2 mutations (*N424fs, K426fs*) were harbored by both MTZ-R and MTZ-S isolates. None of the missense mutations in the *frxA* and *fdxB* genes were associated with resistant phenotype.

A total of 12 missense mutations were identified in the *fur* gene, of which *C150Y* and *N118Q* predominated, however, none of mutations were associated with MTZ-R phenotype . Furthermore, no frameshift or nonsense mutations were detected in the *fur* gene among the studied isolates.

Discussion

The steady increase in *H. pylori* antibiotic resistance worldwide significantly impacts the effectiveness of eradication therapy regimens. Meta-analysis data from Russia (2011–2020) indicate that the most significant rise in *H. pylori* resistance was observed for MTZ (33.95%) and LVX (20.0%) [15]. However, subsequent studies from 2015–2019 and 2020–2024 revealed a slight decrease in LVX resistance from 18.3% to 17.1% [15, 16]. Nevertheless, it must be acknowledged that data on the levels and prevalence of antibiotic resistance are still lacking for most Russian regions [2].

Our study, which included 43 *H. pylori* clinical isolates from St. Petersburg (2014–2022), demonstrated a high level of MTZ resistance — 74.4%. This finding underscores the necessity for a full-scale survey of *H. pylori* resistance in this region and calls into question the efficacy of MTZ in local eradication regimens. In contrast, resistance to LVX (a second- and third-line drug) was lower in our sample, at 27.9%. Notably, among the LVX-R isolates, only 15.0% were monoresistant.

[17]. Summarizing the previously presented data on clarithromycin resistance, 20.9% of *H. pylori* isolates are multidrug-resistant, exhibiting simultaneous resistance to three antibiotics: LVX, MTZ, and clarithromycin [17]. It is well known, that multidrug-resistant *H. pylori* strains represent a major obstacle to successful eradication therapy and pose a significant challenge to global gastroenterological health. Treatment failure rates can reach 30% with single antibiotic resistance and exceed 70% with dual resistance [18, 19]. Since the choice of eradication regimen is empirical, the data obtained highlight the necessity for global, regional, and local monitoring of *H. pylori* antibiotic resistance in our country. They also emphasize the need to adapt treatment strategies in each region based on these data and to implement a rational antibiotic use program in eradication therapy regimens [20].

Given that whole-genome sequencing is the most accurate, reliable, rapid and efficient method for identifying known resistance patterns, as well as searching for new ones, this method was used in the study to identify LVX and MTZ resistance determinants and their association with the phenotypic drug resistance of the Russian *H. pylori* population.

Analysis of the obtained data showed that among all mutations in the *gyrA* and *gyrB* genes, only *D91Y/N/G* in the *gyrA* gene was significantly associated with phenotypic resistance of *H. pylori* to LVX ($p < 0.05$). Another mutation associated with the development of resistance to fluoroquinolones, *N87K*, was detected in only 16.7% of resistant isolates in our study ($p > 0.05$). Nevertheless, considering the low detection frequency of the *D91Y/N/G* mutation, as well as the absence of *D91* and *N87* mutations in LVX-S isolates, the combined detection of *D91Y/N/G* and *N87K* mutations should be considered a more reliable predictor of *H. pylori* resistance to LVX. On the other hand, given that 50.0% of LVX-resistant strains lack resistance markers in their genome, genotyping the *gyrA* gene alone may be insufficient to detect phenotypic resistance to LVX, which in turn casts doubt on the rationality of using the *D91+N87 gyrA* genotype as the sole targets when developing PCR tests for determining *H. pylori* antibiotic resistance in clinical practice. It should be noted that mutations *D91* and *N87* were present in the genome of only multidrug-resistant isolates, while these mutations were not detected among LVZ-monoresistant isolates. This could indicate both the existence of other, unstudied resistance mechanisms and the involvement of phenotypic resistance mechanisms, such as changes in the expression levels of efflux systems, biofilm formation and others.Moreover, multidrug-resistant strains may emerge under the selective pressure of combination antibiotic therapy, which promotes the accumulation of mutations, including in the *gyrA* gene, which is associated with resistance to fluoroquinolones. The obtained data require further investigation using an expanded sample of *H. pylori* isolates resistant and susceptible to LVX.

Given the high heterogeneity of *H. pylori* strains, elucidating the mechanisms of MTZ resistance remains a complex challenge. It is generally accepted that *H. pylori* resistance to MTZ is primarily due to the inactivation of the *rdxA* and *frxA* genes, which encode the reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) nitroreductase and flavin nitroreductase, respectively, which catalyze the reduction of MTZ levels within the cell [21]. Numerous international studies have demonstrated that most MTZ-R *H. pylori* strains carry multiple nonsense and/or frameshift mutations, leading to the loss of nitroreductase functional binding sites.

In the current study, mutations causing stop codon loss, premature termination or frameshifts in the *rdxA* gene were found in 21.9% of MTZ-R isolates and were absent in the MTZ-S group. This finding suggests a potential role of this gene in the development of antibiotic

resistance in the Russian *H. pylori* population. According to E.G. Chua et al., the *R16H/C* mutation in the *rdxA* gene is associated with phenotypic resistance of *H. pylori* isolates to MTZ [22]. Our results showed that the *R16C* mutation, as well as the *S108A/P* and *L62V* mutations in the *rdxA* gene, were found only in MTZ-R isolates, with frequencies of 21.9%, 21.9% and 18.7%, respectively. However, due to the uneven distribution of MTZ-R and MTZ-S isolates in our sample (74.4% and 25.6%), the impact of these mutations on the development of phenotypic resistance to MTZ remains to be elucidated.

Similarly, loss-of-function mutations in the *frxA* and *fdxB* genes have been proposed as potential predictors of *H. pylori* phenotypic resistance to MTZ. However, in our study, frameshift mutations in these genes were found in isolates from both resistant and susceptible phenotypic groups. Furthermore, no mutations associated with phenotypic resistance to MTZ were detected in the *fur* gene. These results suggest that *frxA*, *fdxB*, and *fur* genes are unlikely to play a primary role in the development of *H. pylori* resistance to MTZ. Nevertheless, for a more precise understanding of the resistance mechanisms, further research is necessary, including a comprehensive analysis of other potential genetic factors in conjunction with possible synergistic or epistatic interactions.

Research limitations. This study has several limitations. The sample size was limited both numerically and geographically, preventing a comprehensive analysis of *H. pylori* resistance patterns across Russia. Furthermore, an uneven distribution of resistant and susceptible isolates was observed, which significantly complicates the interpretation of the data and reduces the statistical reliability of the results. However, despite these limitations, this study provides important information about the resistance patterns of Russian *H. pylori* clinical isolates and also highlights the necessity for a systematic, nationwide antibiotic resistance monitoring in our country.

Conclusion

Based on whole-genome sequencing data, this study presents the first comprehensive analysis of phenotypic and genotypic resistance to LVX and MTZ in Russian *H. pylori* clinical isolates. Our results have demonstrated a high prevalence of resistance to both MTZ and LVX, alongside a high frequency of polyresistant isolates resistant to three antibiotics (LVX + MTZ + clarithromycin). Despite the limited sample size, we were able to confirm the key role of *D91* and *N87* mutations in the *gyrA* gene in the development of *H. pylori* resistance to LVX. However, the high frequency of resistant isolates that do not carry known resistance determinants calls into question the effectiveness of current and emerging molecular diagnostic tests for determining *H. pylori* susceptibility and dictates the necessity for larger-scale studies to elucidate the full spectrum of antibiotic resistance mechanisms in *H. pylori* .

Our findings emphasize the exceptional importance of issues related to the continuous monitoring for *H. pylori* antibiotic resistance in our country, as well as tracking genome dynamics and resistance development mechanisms. Such efforts could become a prerequisite for optimizing eradication therapy regimens and improving treatment effectiveness for *H. pylori* infections across different regions of Russia. .

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Stabilization of tigecycline solutions during susceptibility testing of microorganisms by broth microdilution method

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Abstract

Introduction. Tigecycline is widely used in the treatment of infections, leading to the emergence of resistant bacteria. Determining susceptibility by broth microdilution method is recommended to be conducted using freshly prepared Mueller–Hinton broth (MHB) or MHB with antioxidant additives, due to the oxidation of the antibiotic. At the same time, there is no information on the possibility of storing and further using antibiotic solutions.

The aim of the study is to determine the feasibility of stabilizing and rationally using tigecycline solutions to achieve acceptable values of minimum inhibitory concentrations (MIC) when testing control strains by the reference method.

Materials and methods. In the study, the MIC of tigecycline for *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Streptococcus pneumoniae* ATCC 49619 was determined using the microdilution method in MHB, which was pre-prepared and stored for 24–48 hours prior to the study. For the study, a tigecycline stock solution was prepared in water with the addition of various concentrations of Oxyrase, which were stored at 2–6°C and –70°C, and then testing was conducted in accordance with GOST R ISO 20776-1-2022.

Results. The stability of the tigecycline stock solutions does not exceed 5 hours at 2–6°C, but with the addition of 5.0–8.0% Oxyrase, it increases to 16 days, allowing for the determination of MIC values for all test strains within the acceptable range. Changing the storage temperature to –70°C increases the stability of the solutions to 43 days, and with the addition of 5.0–8.0% Oxyrase, to at least 48 weeks

Conclusion. The possibility of stabilizing tigecycline solutions stored at negative temperatures (with and without the addition of Oxyrase) has been determined to obtain acceptable MIC values when determining the susceptibility of control strains to antimicrobial agents. The use of these solutions allowed for a reduction in testing costs through the rational use of the antibiotic.

Keywords: *tigecycline, susceptibility testing, broth microdilution method, Oxyrase, Mueller–Hinton broth*

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Оригинальное исследование
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Стабилизация растворов тигециклина при тестировании чувствительности микроорганизмов методом микроразведений в бульоне

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Аннотация

Введение. Тигециклин широко используется при лечении инфекций, что приводит к появлению устойчивых к нему бактерий. Определение чувствительности методом микроразведений в бульоне рекомендо-

вано проводить, используя свежеприготовленный бульон Мюллера–Хинтон (МХБ) или МХБ с антиоксидантными добавками, что обусловлено окислением антибиотика. Вместе с тем отсутствует информация о возможности хранения и дальнейшего использования растворов антибиотика.

Цель исследования **—** определить возможность стабилизации и рационального использования растворов тигециклина для получения допустимых значений минимальных подавляющих концентраций (МПК) при тестировании контрольных штаммов референтным методом.

Материалы и методы. В работе определяли МПК тигециклина для *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 49619 методом микроразведений в МХБ, заранее приготовленном и хранившемся 24–48 ч до исследования. Для исследования готовили базовый раствор тигециклина в воде и с добавлением различных концентраций оксиразы, которые хранили при 2–6°С и –70°С и затем проводили тестирование в соответствии с ГОСТ Р ИСО 20776-1-2022.

Результаты. В ходе исследования установлено, что при 2–6°С стабильность базовых растворов тигециклина сохраняется не более 5 ч, а с добавлением 5,0–8,0% оксиразы увеличивается до 16 сут, что позволяет получать значения МПК для всех тест-штаммов в допустимом диапазоне. Изменение температуры хранения до –70°С увеличивает стабильность растворов до 43 сут, а с добавлением 5,0–8,0% оксиразы — минимум до 48 нед.

Заключение. Определена возможность стабилизации растворов тигециклина, хранившихся при отрицательных температурах (с добавлением оксиразы и без неё) для получения допустимых значений МПК при определении чувствительности контрольных штаммов к антимикробным препаратам. Применение данных растворов позволило снизить затраты на тестирование за счёт рационального использования антибиотика.

Ключевые слова: *тигециклин, определение чувствительности, метод микроразведений в бульоне, оксираза, бульон Мюллера–Хинтон.*

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Introduction

Microorganisms resistant to antimicrobial agents are one of the greatest threats to global health and food product safety [1]. The rapid increase in the spread of Gram-negative microorganisms, especially those belonging to *Enterobacterales* and *Acinetobacter* spp., which exhibit resistance to the first-line carbapenem drugs, threatens to reduce their effectiveness. For the treatment of infections caused by such strains of microorganisms, colistin and tigecycline are mainly used [2, 3]. However, currently, in more than 40 countries, including Russia, the global spread of resistance in the studied strains to colistin, mediated by the *mcr* gene (mobilized colistin resistance), has been recorded, which significantly reduces its clinical effectiveness [4–7]. Therefore, tigecycline is a critically important drug for medicine[1], especially in the treatment of infections caused by multidrug-resistant microorganisms [8].

Tigecycline is an antibiotic of the new class of glycylcyclines, with a broad spectrum of activity, structurally similar to tetracyclines, but more active against most gram-positive and gram-negative strains of microorganisms. However, certain bacteria, such as *Morganella* spp., *Proteus* spp., *Providencia* spp. and *Pseudomonas aeruginosa*, exhibit natural resistance to tigecycline [9]. Reduced susceptibility of *A. baumannii* to the antibiotic has also been described [10]. Acquired resistance to tigecycline can develop in *Klebsiella pneumoniae*, *E. aerogenes*, *E. cloacae* and others, due to the overexpression of the non-specific efflux gene ArcAB, which confers resistance to many drugs [11].

For the selection and adjustment of antibacterial therapy, it is recommended to conduct microbiological diagnostics with the identification of the pathogen and determination of its susceptibility to tigecycline.

The reference method for determining susceptibility is the broth microdilution method, which allows for the determination of the minimum inhibitory concentrations (MIC) of the tested antibiotics. During the establishment of acceptable values for the MIC of tigecycline for control strains, discrepancies in MIC values were identified, which were associated with the storage period of the Mueller–Hinton broth (MHB). The results of several studies showed that when testing tigecycline in freshly prepared (less than 12 hours before testing) MHB, it was 2–3 times more active against 3 control

[1] WHO. Critically important antimicrobials for human medicine (6th revision ed.) URL: https://www.who.int/publications/i/item/9789241515528 (data of access: 03.03.2025).

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strains (*E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212) than in stored medium (MIC 0.03–0.25 and 0.12–0.50 μg/mL, respectively) [12–14]. In this regard, international standards CLSI[2], EUCAST[3] and Russian recommendations[4] for determining the MIC values of tigecycline using the broth microdilution method recommend using only freshly prepared MHB.

The instability of tigecycline in aqueous solutions is related to its chemical structure, as it can be subjected to two chemical processes that lead to the formation of pharmacologically inactive products. On one hand, the phenolic group of tigecycline makes it susceptibility to oxidation, especially at pH values exceeding 7.0; on the other hand, at lower pH values, tigecycline is more prone to non-enzymatic epimerization[5].

For therapeutic purposes, tigecycline is released in the form of a lyophilized powder[6], containing excipients such as lactose to stabilize the drug against epimerization, and hydrochloric acid or sodium hydroxide to adjust the pH in the range of 4.5–5.5 and to prevent oxidation [10, 15].

Since the pH of the MHB does not change over time, the discrepancies in the MIC results are attributed to the accelerated oxidative degradation of tigecycline caused by the increased amount of dissolved oxygen in the MHB during storage. To stabilize tigecycline, various antioxidant additives in MHB were investigated, such as: ascorbic acid, sodium pyruvate, sodium thioglycolate, L-cysteine, catalase, as well as anaerobic storage conditions of MHB [12, 15, 16]. The addition of ascorbic acid and pyruvate at concentrations of 0.3% and 6%, respectively, contributed to the stabilization of tigecycline for up to 7 days. However, the study [16] showed that ascorbic acid causes rapid degradation of tigecycline and leads to a loss of antibacterial activity. In several publications, the possibility of using Oxyrase — an enzyme that helps reduce oxygen concentration in MHB—has been investigated [12, 14, 16]. The results of the studies showed that tigecycline activity was maintained in a 2% Oxyrase solution for up to 7 days at 4–6°C.

The study [12] describes the freezing of MHB containing tigecycline and dispensed into 96-well plates at –20°C. Testing the susceptibility of test strains after thawing showed that the antibacterial activity was preserved for up to 6 weeks, and the test results matched those obtained with freshly prepared MHB.

Analysis of publications showed that all manipulations in the studies (antioxidant additives, storage in frozen state, in anaerobic conditions) were carried out with MHB, therefore, it seems important to study the possibility of storing specifically aqueous solutions of the antibiotic.

In accordance with the requirements of GOST R ISO 20776-1-2022[7], which regulates the procedure for performing the broth microdilution method, antibiotic stock solutions are used for testing, a portion of which, as a rule, remains unused. According to the requirements of GOST R ISO 20776-1-2022, the storage of stable antibiotic solutions is permitted, while the storage of unstable ones, such as aqueous tigecycline solutions, is not regulated in any way. It is only specified that if there is no information on the stability of the solutions under certain storage conditions, a fresh stock solution should be prepared for each batch being tested.

The aim of the study is to determine the possibility of stabilizing and rationally using tigecycline solutions to achieve acceptable MIC values when testing control strains by the reference method.

Materials and methods

In the study, the chemical substances tigecycline (Sigma) and Oxyrase (Sigma) were used. Determination of the MIC values of tigecycline for the control (test) strains *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *S. pneumoniae* ATCC 49619 was conducted using the microdilution method in MHB (SRCAMB), which was prepared in advance and stored for 24–48 hours at room temperature before the study. When working with the test strain *S. pneumoniae* ATCC 49619, 5% lysed horse blood and 20 mg/L β-NAD (Sigma) were added to the broth. All strains used in the study were obtained in lyophilized form from the State Collection of Pathogenic Microorganisms of SCPM-Obolensk.

Lysed horse blood was prepared from defibrinated blood (ECOlab). In order to carry out the preparation, sterile deionized water was added to the defibrinated

[2] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Thirty-third Informational Supplement. CLSI document M100, 33rd Edition. USA; 2022. 402 p.

[3] European Committee for Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters (Version 15.0). URL: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_15.0_Breakpoint_Tables.pdf (data of access: 12.03.2025).

[4] Russian recommendations "Determination of the sensitivity of microorganisms to antimicrobial drugs" (version 2024-02). URL: https://www.antibiotic.ru/files/334/ocmap2024.pdf

[5] Fawzi M.B., Zhu T., Shah S.M. Tigecycline compositions and methods of preparation (patent). United States US-8975242-B2. 2011. URL: https://patentimages.storage.googleapis.com/c2/25/07/73a12f6c15cdfa/US8975242.pdf (data of access: 07.03.2025).

[6] Directory of medicines. Tigacil. Instructions for use. 2024. (In Russ.) URL: https://www.vidal.ru/drugs/tigacil__23094 (data of access: 12.03.2025).

[7] GOST R ISO 20776-1-2022 Study of the sensitivity of infectious agents and evaluation of the functional characteristics of products for the study of sensitivity to antimicrobial agents. Part 1. A reference method of microdilution in broth for laboratory investigation of the activity of antimicrobial agents against fast-growing aerobic bacteria that cause infectious diseases. 2022. 20 p. (In Russ.)

horse blood in a 1:1 ratio, and it was placed in a freezer for 7–8 hours at –20°C. Then, the thawed blood at room temperature was subjected to repeated freeze-thaw cycles, repeating this process 4 times until the complete lysis of the blood cells. After that, the lysed horse blood was clarified by centrifugation at 7000 rpm for 30 minutes on an Eppendorf Centrifuge 5702 (Eppendorf).

Testing was conducted thrice in accordance with the requirements of GOST R ISO 20776-1-2022. To carry this process out, a stock solution of tigecycline with a concentration of 512 mg/L was prepared in water and in water with the addition of various concentrations of Oxyrase (2.0, 3.0, 5.0, and 8.0%), stored at 2–6°C in a household refrigerator and at –70°C in an MDF-U33V low-temperature freezer (Sanyo). Every hour, individual aliquots of the solution were removed from the storage chamber, thawed at room temperature for 20–30 minutes, prepared as working (twofold) dilutions in distilled water in the concentration range of 0.016–1.000 mg/L, and filled into a 96-well plate.

Inoculates of all test strains were prepared in a saline solution with a concentration of $1–2 \times 10^8$ CFU/mL and 0.1 mL was added to 9.9 mL of 2-fold concentrated MHB. Inoculates in MHB were added in a 1:1 ratio to prepared plates, and were incubated at 35 ± 1°C for 18 ± 2 hours, with plates containing *S. pneumoniae* ATCC 49619 incubated in an atmosphere of 4–6% CO_2. The results were recorded according to the EUCAST methodology.

The results were processed using the MS Excel software package. The reliability of various averages was assessed using the Student's t-test. The two-tailed Fisher criterion was used for comparative analysis. Differences were considered significant at $p < 0.05$.

Results

The MIC of tigecycline for all test strains using stock solutions stored at 2–6°C for up to 3 hours corresponded to the target values, and with further storage up to 5 hours, they fell within the acceptable range (**Table 1**). Further, with an increase in storage time, the MIC values exceeded the acceptable range.

The MIC values of tigecycline for all test strains using antibiotic stock solutions stored at -70°C for up to 31 days corresponded to the target values, and with further storage up to 43 days, it fell within acceptable ranges (**Table 2**). Longer storage (≥ 44 days) resulted in MIC values for all 4 test strains exceeding the acceptable ranges.

The results obtained in the first stage of the study showed that the activity of the stock solutions of tigecycline was maintained for a longer period at –70°C (up to 43 days) than at 2–6°C (no more than 5 hours), which ensured the determination of the minimum inhibitory concentration (MIC) of tigecycline for all test strains within acceptable value ranges.

The second stage of the study is dedicated to examining the effect of adding Oxyrase on the MIC of tigecycline for 4 test strains. Increasing the concentration of Oxyrase contributed to the longer storage of tigecycline stock solutions at –2–6°C compared to solutions without a stabilizer, as the obtained MIC values for the tested strains corresponded to acceptable values for a longer period (**Figure**, **Table 3**). The addition of Oxyrase at concentrations of 5% and 8% to the tigecycline stock solution increased its storage time at 2–6°C from 4–5 hours to 16 days and ensured the attainment of acceptable MIC values for 4 test strains.

The dependence of tigecycline MIC for *E. coli* ATCC 25922 (*a*), *S. aureus* ATCC 29213 (*b*), *E. faecalis* ATCC 29212 (*c*), and *S. pneumoniae* ATCC 49619 (*d*) on the concentration of Oxyrase added to the antibiotic stock solutions and their storage periods at 2–6°C.

The results of the tigecycline MIC measurements for test strains, obtained using stabilized Oxyrase antibiotic stock solutions during storage at –70°C, are presented in **Table 4**. For convenience, the MIC results are presented in arbitrary units: average MIC values from 3 measurements within the acceptable range were marked as C (Correct); average values from

Table 1. Effect of storage time at 2–6°C of the tigecycline stock solution on the MIC value, mg/L

Storage time of the tigecycline stock solution	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*S. pneumoniae* ATCC 49619
0 h (control)	0.06–0.12	0.06–0.12	0.03–0.06	0.03–0.06
1–2 h	0.06–0.12	0.06–0.12	0.03–0.06	0.03–0.06
3 h	0.06–0.12	0.06–0.12	0.03–0.06	0.03–0.06
4 h	0.12–0.25	0.12–0.25	0.03–0.12	0.06–0.12
5 h	0.12–0.25	0.12–0.25	0.06–0.12	0.06–0.12
6 h	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0
7 h	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0
8–10 h	≥ 1.0	≥ 1.0	≥ 1.0	≥ 1.0
Acceptable range, mg/L	0.03–0.25	0.03–0.25	0.03–0.12	0.016–0.12
Target values, mg/L	0.06–0.12	0.06–0.12	0.06	0.03–0.06

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Table 2. Effect of storage time at –70°C of tigecycline stock solutions on the MIC value, mg/L

Storage time of tigecycline solutions	*E. coli* ATCC 25922	*S. aureus* ATCC 29213	*E. faecalis* ATCC 29212	*S. pneumoniae* ATCC 49619
1–18 days	0.06–0.12	0.06–0.12	0.03–0.06	0.03–0.06
19–31 days	0.06–0.12	0.06–0.12	0.03–0.06	0.03–0.06
32–43 days	0.12–0.25	0.12–0.25	0.06–0.12	0.06–0.12
44–56 days	0.5–1.0	0.5–1.0	0.25–0.50	0.25–0.50
57–68 days	0.5–1.0	0.5–1.0	0.25–0.50	0.25–0.50
69–80 days	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0
≥ 81 days	≥ 1.0	≥ 1.0	≥ 1.0	≥ 1.0
Acceptable range, mg/L	0.03–0.25	0.03–0.25	0.03–0.12	0.016–0.120
Target values, mg/L	0.06–0.12	0.06–0.12	0.06	0.03–0.06

3 measurements exceeding the acceptable range were marked as HE (High error).

As seen from Table 4, the use of Oxyrase as a stabilizer for tigecycline stock solutions showed that the stability of these solutions is maintained up to 18 weeks with the addition of 2% Oxyrase, up to 28 weeks with the addition of 3% Oxyrase, and up to 48 weeks with the addition of 5 or 8% Oxyrase, resulting in MIC values for tigecycline for all tested strains corresponding to acceptable levels (C).

Discussion

Currently, tigecycline is the drug of choice for treating many severe infections caused by multidrug-resistant microorganisms. Due to its broad-spectrum antibacterial activity, tigecycline is often used empirically, which leads to the emergence of resistant bacteria [17].

Therefore, the treatment of infectious diseases, including tigecycline, should be based on the results of pathogen identification and antimicrobial susceptibility testing.

Determining susceptibility to tigecycline using broth microdilution methods is associated with the problem of its instability in aqueous solutions. To obtain reliable test results according to CLSI and EUCAST standards, it is recommended to use freshly prepared MHB.

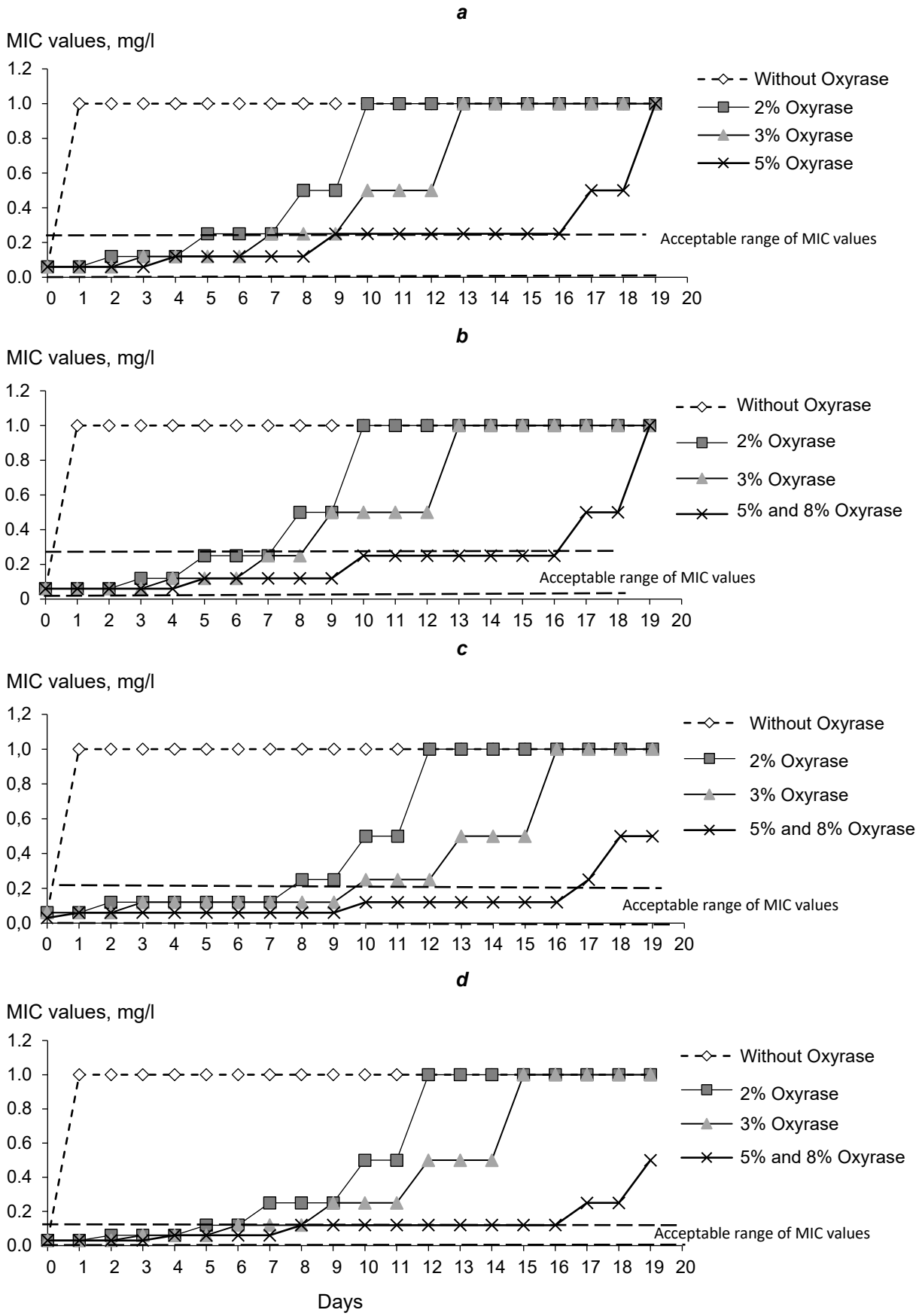
This study examined the effect of various methods for stabilizing basic aqueous solutions of tigecycline: by altering storage temperature and using Oxyrase stabilizer in different concentrations for 4 strains,

Table 3. Storage periods (days) of tigecycline stock solutions with different Oxyrase concentrations at 2–6°C while maintaining acceptable MIC values

Test strains	Without Oxyrase	Oxyrase concentration, %			
		2	3	5	8
E. coli ATCC 25922	5 ч	7	9	16	16
S. aureus ATCC 29213	5 ч	7	8	16	16
E. faecalis ATCC 29212	4 ч	7	9	16	16
S. pneumoniae ATCC 49619	5 ч	6	8	16	16

Table 4. Conditional tigecycline MIC values for test strains Oxyrase concentration in antibiotic stock solutions and storage duration at –70°C

Storage time of stock solutions, weeks	*E. coli* ATCC 25922				*S. aureus* ATCC 29213				*E. faecalis* ATCC 29212				*S. pneumoniae* ATCC 49619			
	2%	3%	5%	8%	2%	3%	5%	8%	2%	3%	5%	8%	2%	3%	5%	8%
0	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
12	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
18	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
28	HE	C	C	C	HE	C	C	C	HE	C	C	C	HE	C	C	C
48	HE	HE	C	C	HE	HE	C	C	HE	HE	C	C	HE	HE	C	C



The dependence of tigecycline MIC values for *E. coli* ATCC 25922 (*a*), *S. aureus* ATCC 29213 (*b*), *E. faecalis* ATCC 29212 (*c*), and *S. pneumoniae* ATCC 49619 (*d*) on the concentration of oxyrase added to the antibiotic stock solutions and their storage periods at 2–6°C.

including a fastidious strain *S. pneumoniae* ATCC 49619.

In the course of the study, pre-prepared MHB was used and stored for 24–48 hours. It has been determined that at 2–6°C, the stability of tigecycline stock solutions without Oxyrase is maintained for no more than 5 hours, while with 5% and 8% Oxyrase, it increases to 16 days. When these solutions are stored without Oxyrase at –70°C, their stability increases to 43 days, and with the addition of 5% and 8% Oxyrase, it increases to at least 48 weeks. The obtained results correlate with published data [12, 14, 16], which show that the activity of tigecycline in MHB is maintained for up to 6 weeks (42 days) at –17–18°C, and at 4–6°C with the addition of 2% Oxyrase solutions, it is maintained for up to 7 days (see Table 3).

In several publications, the authors note that Oxyrase and chemically pure tigecycline are quite expensive products [12–14]. The cost calculations showed that from 5 mg of chemically pure tigecycline (priced at an average of 45,000 rubles/5 mg), more than 200 plates can be prepared with a working concentration range of tigecycline (0.06–4.00) mg/L. At the same time, the cost of preparing one plates will be approximately 270 rubles (including the cost of the plates itself)[8]. The broth microdilution method is a labor-intensive process, so not every bacteriological laboratory can use such a large number of plates in a single experiment. Unused tigecycline stock solution can be aliquoted into cryovials and stored at negative temperatures until the next use, and the addition of Oxyrase will further extend the storage time and the possibility of using MHB stored for 24–48 hours. The cost of the same number of plates with the addition of 5% Oxyrase increases slightly (on average by 7–10%).

Conclusion

The possibility of stabilizing tigecycline solutions stored at negative temperatures (with and without the addition of Oxyrase) has been determined to obtain acceptable MIC values when determining the susceptibility of control strains to antimicrobial agents. The use of these solutions made it possible to reduce testing costs through the rational use of the antibiotic. In the future, work will continue on studying the possibility of using these tigecycline solutions in the testing of clinical strains.

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REVIEWS

Review
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Application of the pseudovirus-based neutralization assay in the search for new antiviral drugs

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Abstract

In recent years, significant progress has been made in the field of drug development, particularly due to the use of computer modeling methods. One of the key stages in the development of new antiviral drugs is testing the efficacy of promising candidates in *in vitro* experiments using target viruses. The application of new technologies for conducting primary screening with pseudotyped viruses simplifies research, increases its efficiency and ensures the biosafety of the conducted studies.

The aim of this review is to analyze previous studies that have demonstrated the successful use of pseudovirus technology for the search of new chemotherapeutic agents against a range of RNA-containing viruses.

The analysis involved the literature presented in the PubMed, Scopus, Elsevier, and Google Scholar databases as of March 1, 2025. For the search, the following keywords were used: pseudovirus, virus inhibition, antiviral drugs, RNA viruses.

Pseudotyped viruses are recombinant viral particles that have the core proteins of one virus and the surface proteins of another, studied virus. The advantages of pseudovirus technology are its safety, high level of reproducibility of results, and the possibility of standardization. The lentivirus-based system was one of the first to be developed and remains one of the most in-demand. Using pseudoviruses, candidate molecules for infections caused by RNA-containing viruses, such as HIV-1, hepatitis C virus, tick-borne encephalitis virus, avian influenza viruses, and SARS-CoV-2, have been selected and studied. Most of the selected drugs act at the initial stage of the virus entry into the target cell. The examples provided illustrate the significant contribution of pseudovirus technology in dealing with serious socially significant diseases caused by RNA-containing viruses.

Keywords: *pseudoviruses, antiviral drugs, HIV-1, orthoflaviviruses, influenza virus, review*

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Использование метода нейтрализации псевдовирусов для поиска новых антивирусных препаратов

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Аннотация

В последние годы в области разработки лекарственных препаратов достигнут большой прогресс, в частности, благодаря использованию методов компьютерного моделирования. Одним из ключевых этапов разработки новых антивирусных препаратов является проверка эффективности перспективных кандида-

тов в экспериментах *in vitro* с использованием вирусов-мишеней. Использование новых технологий для проведения первичного скрининга с применением псевдотипированных вирусов обеспечивает упрощение исследований, повышение их эффективности и соблюдение биобезопасности проводимых исследований. **Целью** данной работы является анализ исследований, в которых продемонстрировано успешное использование псевдовирусной технологии для поиска новых химиопрепаратов против ряда РНК-содержащих вирусов.

При подготовке обзора был проведён анализ литературы, представленной в базах PubMed, Scopus, Elsevier, Google Scholar по состоянию на 01.03.2025. Для поиска использовали ключевые слова: pseudovirus, virus inhibition, antiviral drugs, RNA viruses, псевдовирус, ингибирование вируса, противовирусные препараты, РНК-содержащие вирусы.

Псевдотипированные вирусы представляют собой рекомбинантные вирусные частицы, которые имеют коровые белки одного вируса, а поверхностные белки — другого, исследуемого вируса. Достоинствами псевдовирусной технологии являются её безопасность, высокий уровень воспроизводимости результатов и возможность стандартизации. Система, основанная на лентивирусах, была разработана одной из первых и по-прежнему является одной из наиболее востребованных. С помощью псевдовирусов были отобраны и исследованы молекулы-кандидаты для инфекций, вызываемых РНК-содержащими вирусами, такими как ВИЧ-1, вирус гепатита С, вирус клещевого энцефалита, вирусы гриппа птиц, SARS-CoV-2. Большинство из отобранных препаратов действуют на начальном этапе проникновения вируса в клетку-мишень. Приведённые примеры иллюстрируют существенный вклад технологии псевдовирусов в борьбу с серьёзными социально значимыми заболеваниями, вызываемыми РНК-содержащими вирусами.

Ключевые слова: *псевдовирусы, антивирусные препараты, ВИЧ-1, ортофлавивирусы, вирус гриппа, обзор*

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Introduction

The search and development of new drugs for the prevention and treatment of viral infections is one of the most important objectives in medical chemistry, biology and medicine due to the widespread prevalence of various socially dangerous viral infections and the constant emergence of new viral diseases [1, 2].

Progress in the development of pharmaceuticals in recent years has largely been made possible by the application of computer modeling methods to predict the structure of target molecules and their interactions with candidate drug compounds [3]. The awarding of the Nobel Prize in 2024 for the development of methods for computer-aided protein design and prediction of protein quaternary structure vividly illustrates the significance of scientific achievements in this field of knowledge. In a short period, more than 200 million protein structures were deciphered (predicted) using the AlphaFold2 program, involving approximately 2 million researchers from 190 countries[1]. Recent advancements in artificial intelligence also create fundamentally new opportunities for designing new drugs based on computer-aided design of candidate drug compounds and target proteins [3].

At the same time, the promising candidates for future antiviral drugs selected using computer technologies need to be tested in real *in vitro* experiments, followed by *in vivo* testing of the selected active molecules. Among socially significant infectious diseases, it is important to note infections caused by various viral agents such as HIV-1, hepatitis C, various pathogenic orthoflaviviruses, influenza viruses, SARS-CoV-2, as well as many other dangerous and particularly dangerous infections. Experimental work with these pathogens requires special working conditions to meet strict biosafety requirements and is characterized by the complexity of conducting laboratory experiments, the lack of simple and safe laboratory methods for working with infectious agents, and the inability to cultivate multiple viruses in the laboratory, which makes conducting experiments complicated or even impossible. A fundamentally important alternative for primary screening is the use of pseudotyped viruses [4]. The use of replication-deficient pseudoviruses carrying viral envelope proteins is a safe and useful method widely employed by virologists for studying, screening and developing new antiviral chemotherapeutics.

The aim of the review is to analyze studies that have demonstrated the successful use of pseudovirus

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technology for the search of new chemotherapeutic agents against a range of RNA-containing viruses.

The analysis included literature presented in the scientific databases PubMed, Scopus, Elsevier, and Google Scholar as of March 1, 2025. For the search, the following keywords were used: pseudovirus, virus inhibition, antiviral drugs, RNA viruses, псевдовирус, ингибирование вируса, противовирусные препараты, РНК-содержащие вирусы.

In the PubMed scientific electronic database, a search using a combination of keywords found 293 sources, of which 228 had full text available in open access. Similarly, the search was also conducted using the Scopus, Elsevier and Google Scholar scientific databases, but for these, the publication date of the articles was limited to 2023–2024 in order not to overlook the most relevant research. Overall, during the literature search in the listed databases in Russian and English, conducted with consideration of selection criteria such as publication year and availability of publications for reading, approximately 1700 sources relevant to the topic were analyzed. Due to the volume limitation for the study, 68 of those sources were selected.

Pseudoviruses

Pseudoviruses are artificially created viruses with defective genomes that, due to similar conformational structures of surface glycoproteins, are capable of entering susceptible cells just like natural viruses do [5–7]. Unlike viruses, pseudoviruses can usually replicate only during a single replication cycle [7, 8]. The replication limitation makes conducting experiments with them safe and provides new opportunities for studying highly pathogenic RNA viruses. It is important to note that the use of pseudoviruses allows for research even in cases where natural viruses are impossible or very difficult to cultivate in laboratory conditions [9].

Once again, it is important to emphasize that, unlike infectious viruses, working with pseudoviruses is safe because changes (mutations) have been incorporated into the coding regions of the genome, limiting the virus's development to just one replication cycle. Therefore, pseudoviruses are often referred to as single-cycle infection viruses. As a rule, pseudotyped viruses carry marker genes (such as luciferase or GFP), which allows for easier and more accurate quantitative assessment in experiments with them.

Thus, the advantages of pseudovirus technology are safety, a high level of reproducibility of results, the possibilities of standardization and the generation of new pseudovirus variants for further research development. This predetermines the widespread use of this technology by researchers to study the characteristics of virus entry into target cells, determine the presence of virus-neutralizing antibodies, as well as search for and develop new antiviral drugs.

Features of pseudovirus construction

Most commonly, pseudoviruses are divided into three main types: those with a lentiviral genome (HIV-1), those with a vesicular stomatitis virus genome and those with a mouse leukemia virus genome [10]. The system based on lentiviruses, particularly HIV-1 Env-pseudoviruses, was one of the first developed for analyzing the immune response to HIV-1 vaccines and searching for antiviral drugs [11]. It remains one of the most sought-after and frequently used in research.

Env-pseudoviruses of HIV-1 are recombinant viral particles obtained by transfecting eukaryotic cells with two plasmids: a core plasmid and an envelope plasmid. The core plasmid contains the genes of structural, regulatory, and accessory proteins of HIV-1 necessary for the assembly of viral particles, as well as sequences required for packaging the viral RNA (Ψ); the envelope plasmid, in turn, carries the gene of the surface glycoprotein (*env*) of a specific subtype of HIV-1. As a result of transfection, viral particles with a defective genome are formed, which are unable to ensure the assembly of infectious progeny virions upon infection [12, 13]. When working with HIV-1 Env-pseudoviruses, the genetically modified TZM-bl cell line, a derivative of the HeLa cell line, is used. This cell line expresses CD4 receptors and the CCR5 and CXCR4 co-receptors on its surface, which HIV-1 uses to enter target cells. An important feature of the TZM-bl cell line is the presence of integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under the transcriptional control of the long terminal repeat of HIV-1, the protein products of which are used to detect the entry of HIV-1 Env-pseudoviruses into target cells. Thus, upon the entry of the pseudovirus into the TZM-bl target cell, the expression of reporter genes is initiated in response to the synthesis of the viral protein Tat, which, for example, for the luciferase gene, is detected using a luminometer. In this case, a high intensity of luminescence corresponds to the entry of pseudovirus particles into target cells, while a suppression of luminescence, on the contrary, indicates the neutralization of HIV-1 Env pseudoviruses [14, 15]. The **Figure** illustrates the general principle of operation of HIV-1 Env-pseudoviruses.

By replacing the *env* gene in the lentiviral system with the envelope protein coding genes of other viruses, it is possible to obtain lentiviral particles that display the corresponding viral proteins on their surface. Using this technology, pseudoviruses of enveloped RNA viruses such as influenza viruses [16], coronaviruses [3], retroviruses [17], flaviviruses [18] and others have been obtained, which are successfully used in research.

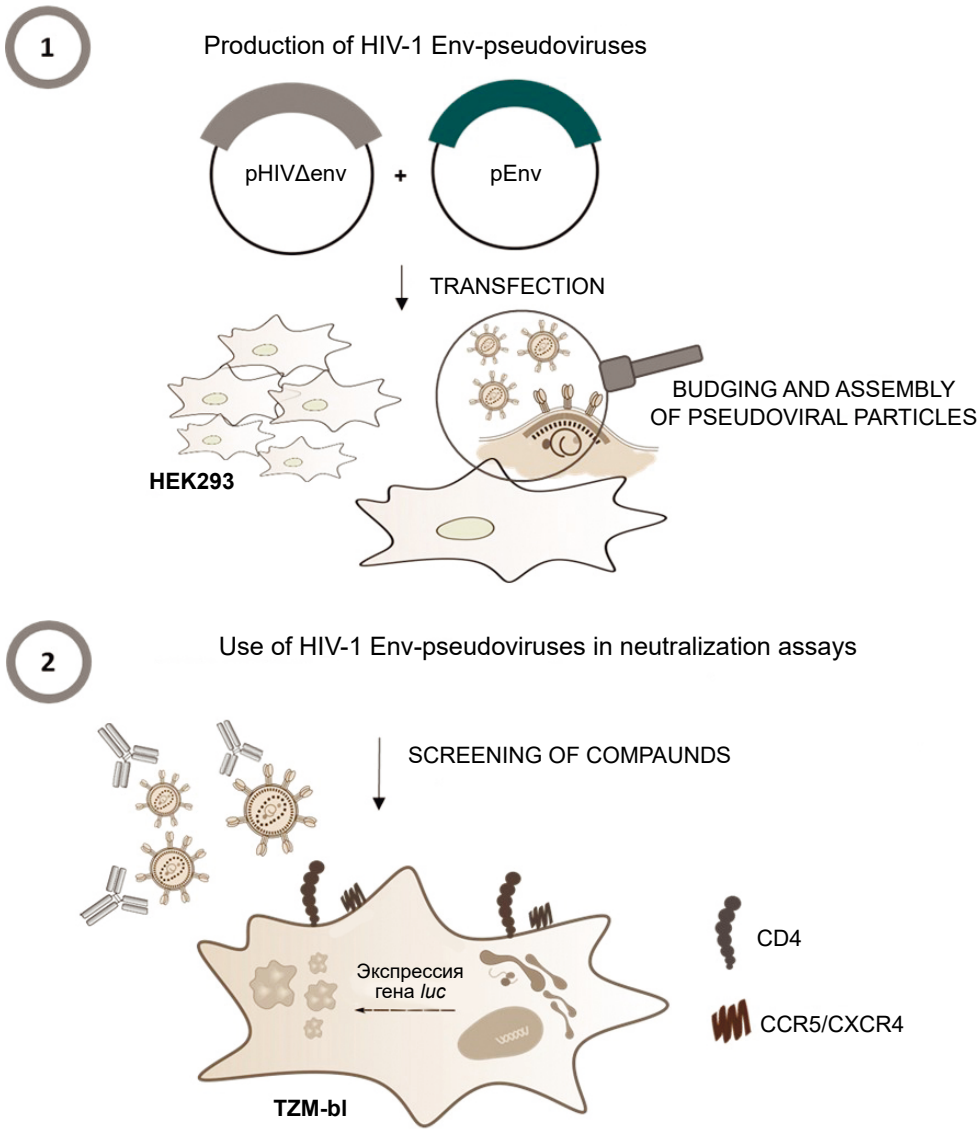
The pseudovirus platform can equally be useful for studying the entry of viruses that rapidly accumulate mutations due to adaptation in culture, due to the absence of selective pressure such as host immune re-

sponses that exist *in vivo*. For example, *in vitro* studies using live HIV-1 led to mutations in the Env glycoprotein due to the error-prone nature of reverse transcriptase, which facilitated adaptation to tissue culture and resulted in phenotypic changes. As a result, the entry of tissue culture-adapted HIV-1 strains into cells did not correspond to what occurs during natural infection in humans and, thus, did not provide a clinically relevant model [20].

It should be noted that, despite the aforementioned advantages, the pseudovirus system has a number of limitations that should be considered when conducting any research. This system is primarily designed to simulate the process of viral agent entry into a cell *in vitro*; however, the process of proliferation and release of viral particles cannot be modeled. The distribution, conformation, and density of heterologous viral glycoproteins on pseudoviruses do not always reflect their natural state on the surface of native viral particles [13].

Therefore, the results of analyses using pseudotyped viruses do not always align with data obtained through analyses using native viruses [13]. Finally, the use of pseudoviruses is effective in studying enveloped viruses. For viruses such as rotavirus and poliovirus, the pseudovirus system functions unsatisfactorily [7]. To address the latter issue, a self-organizing pseudovirus system was developed, but it will not be considered here.

This review will analyze and summarize the results of using pseudovirus technology to search for drugs against HIV-1, orthoflaviviruses and avian influenza viruses.



The general principle of operation of HIV-1 Env-pseudoviruses [29, with added changes].

Practical work with HIV-1 Env-pseudoviruses includes two main stages: Stage 1 — assembly of viral particles using transfection of the HEK293 cell line with two plasmids: core (pHIVΔenv) and envelope (pEnv); Stage 2 — neutralization assay using chemotherapeutic agents to determine their ability to block the entry of pseudoviruses into the target cell.

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Pseudoviruses and antiviral drugs

Antiviral drugs that inhibit HIV entry (Riboviria, Pararnavirae, Artverviricota, Revtraviricetes, Ortervirales, Retroviridae, Orthoretrovirinae, Lentivirus)

Currently, the basis of therapy for patients with HIV infection is antiretroviral therapy (ART) — a combination of several antiviral drugs that suppress the replication of the virus by targeting different stages of its life cycle, thereby preserving the immune functions of the body and reducing the risk of virus transmission [21]. However, the spread of HIV resistance to ART reduces the effectiveness of treatment and increases mortality from HIV/AIDS [22].

Targeting the initial stage of the virus's entry into the target cell offers several advantages compared to other stages of the HIV-1 life cycle [23]. Firstly, the viral genetic material cannot integrate into the host cell's genome. Secondly, entry inhibitors do not need to cross the cell membrane, unlike reverse transcriptase, integrase, or protease inhibitors. Thirdly, since the virus entry consists of separate stages, there are several targets for entry inhibitors, which guarantees protection against cross-resistance [24].

An important point in the search for such drugs is that HIV-1 requires co-receptors to enter the cell, which expands the list of targets for antiviral drugs [25]. Among the first effective inhibitors of HIV-1 entry was the drug maraviroc [26]. The drug binds to CCR5, thereby blocking the subsequent stages of viral and cellular membrane fusion and, consequently, the virus's entry into target cells. In this case, HIV-1 cannot enter human macrophages and T-lymphocytes. However, maraviroc can cause serious, life-threatening side effects. This fact once again underscores the necessity for the development of new HIV-1 entry inhibitors.

The pseudovirus technology is widely used to study drugs that block the binding of the virus to membrane receptors and its entry into target cells. The list of such drugs is provided in **Table 1**.

Enfuvirtide (T20), which for a long time remained the only viral fusion inhibitor used in combination therapy for HIV infection, is a peptide drug, but it has relatively low antiviral activity and easily induces drug resistance. The activity of lipopetide inhibitors developed based on T20 is significantly higher. Their development, structural analysis, function, and comparison of activity in suppressing the entry of HIV-1 and pseudovirus into cells are the focus of the research [27].

Another example could be the artificial peptide HNG-105, created by click conjugation of the linear peptide 12p1 (RINNIPWSEAMM). HNG-105 was studied using surface plasmon resonance spectroscopy and pseudovirus inhibition assays. The results show that the HNG-105 molecule may be effective against HIV-1 subtypes and highlight its potential as a lead for the development of therapeutic and microbicidal agents to help combat the spread of AIDS [28].

HIV entry inhibitors have also been developed based on nucleic acids. Thus, 23 clones of RNA aptam-

Table 1. Antiviral drugs against HIV-1, obtained and/or studied using pseudovirus technology

Substance	Mechanism of action	Reference
Lipopeptides based on T20 (enfuvirtide)	Fusion inhibitor	[27]
HNG-105 — modification of the linear peptide 12p1 (RINNIPWSEAMM)	Inhibition of gp120 by interacting with a site distinct from the CD4 or coreceptor binding site, resulting in a sharp decrease in gp120's affinity for either	[28]
RNA aptamers	Binding to glycoproteins gp120 or gp41 and other viral surface molecules	[29]
DNA triplexes with hydrophobic modifications	Interaction with the primary pocket in the repeat of N-heptad of glycoprotein 41 (gp41)	[30]
Thiolated derivatives of pyrimidine	Selective inhibition dependent on the co-receptor preference of the pseudovirus	[31]
Small molecules NBD-14009 and NBD-14010 are analogs of N-phenyl-N'-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide	Inhibition of cell fusion and HIV-1 transmission from cell to cell	[32]
Methylgallate from the edible mushroom *Pholiota adiposa*	Inhibition of HIV-1 replication in TZM-BL cells infected with a pseudovirus, blocking both the virus entry process and the activity of key enzymes necessary for its life cycle	[36]
DAVEI is a recombinant peptide chimera of cyanovirin-N (CVN) lectin and HIV-1 gp41	Binding of gp120 and gp41	[40]
Catechin from *Peltophorum africanum*	Not described	[37]
Methanol extract of *Elaeodendron transvaalense* root	Not described	[37]
Percolation extract of *Spatholobus suberectus Dunn*	Interaction with the viral envelope glycoprotein gp160	[38]
Glycyrrhizin – mixture of derivatives of nicotinic and glycyrrhizic acids	Prevents the virus from entering the target cell	[34]

ers against the pseudotyped virus HIV-1CAP45, which belongs to subtype C viruses endemic to countries in sub-Saharan Africa and responsible for the majority of HIV-1 infections worldwide, were isolated and studied. Aptamers inhibited the infection of target cells by binding to both different glycoprotein molecules (gp120 or gp41) and other viral surface molecules necessary for infection. Thus, they can be used as analytical tools to study the mechanisms of HIV-1 entry and as inhibitors of this process [29]. Another category of HIV-1 inhibitors may consist of complexes based on DNA triplexes with hydrophobic modifications, interacting with the primary pocket in the N-heptad repeat of glycoprotein 41 (gp41). Using Env-pseudoviruses, it has been demonstrated that triplexes are inhibitors of virus-cell fusion [30].

As potential inhibitors of HIV-1 entry, synthetic chemical compounds were also investigated. Thiolated pyrimidine derivatives were synthesized and their antiretroviral effect against human immunodeficiency virus type 1 (HIV-1IIIB) and chimeric HIV-1 pseudovirions was quantitatively determined in viral infectivity assays, including syncytium inhibition assays, as well as single-cycle viral infection assays on HeLaCD4-LTR/β-gal cells. The inhibition was selective and depended on the co-receptor preference of the pseudovirus [31].

Through targeted screening of commercial libraries in 2005, small molecules, analogs of N-phenyl-N'-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide, were identified as a new class of HIV-1 entry inhibitors that blocked the gp120-CD4 interaction [32]. However, they had a number of significant drawbacks. The following decade, this group of scientists systematically studied and modified these substances, ultimately resulting in the small molecules NBD-14009 and NBD-14010, which were tested against a panel of 51 HIV-1 Env-pseudoviruses representing various subtypes of clinical isolates. These compounds exhibited antiviral activity in the nanomolar range ($IC_{50} \approx 150$ nM). They also inhibited cell fusion and the transmission of HIV-1 from cell to cell [33].

Studies dedicated to natural compounds targeting the stage of HIV-1 entry into target cells are of particular interest. The drug Glycyvir — a multi-component mixture containing mono-, di-, tri- and tetranicotinates of glycyrrhizic acid — exhibited pronounced inhibitory activity against HIV-1 pseudoviruses of subtypes B, A6, and the recombinant form CRF63_02A (IC_{50} range 3.9–27.5 μM). Analysis of the inhibitory activity of Glycyvir depending on the timing of its addition to HIV-1 Env-pseudoviruses and TZM-bl cells suggested that this drug acts at the stage of virus entry into the target cell [34]. Later, a modified synthesis method for the glycyrrhizin preparation was developed, which allowed for the replacement of the initial reagents with less toxic and cheaper ones, resulting in a glycyrrhizin preparation with biological activity similar to the original Glycyvir [35].

In the study [36], the antiviral activity of methyl gallate from the edible mushroom *Pholiota adiposa* was demonstrated. Methyl gallate inhibited the entry of Env-pseudovirus into TZM-bl cells, while exhibiting low toxicity towards the cell cultures used [36]. To identify inhibitory substances from plants, an HIV-1 subtype C pseudovirus (HIV-1-C) was created, and as controls, wild-type HIV-1 subtype B pseudoviruses (HIV-1-B) and mutants resistant to nucleoside and non-nucleoside reverse transcriptase inhibitors were used. Thus, catechin obtained from *Peltophorum africanum* inhibited the entry of HIV-1-C and HIV-1-B pseudoviruses with selectivity indices of 6304 μM (IC_{50}: 0.49 μM, CC_{50}: 3089 μM) and 1343 μM (IC_{50}: 2.3 μM, CC_{50}: 3089 μM), respectively [37]. The percolation extract of *Spatholobus suberectus Dunn* (SSP) possesses a broad spectrum of antiviral activity against the entry of SARS-CoV, H5N1 IAV, and HIV-1. In particular, in the case of HIV-1, SSP interacted with the viral envelope glycoprotein gp160, which is responsible for the virus's entry into the host target cells [38].

New antiviral drugs capable of inactivating the infectious activity of viral particles have been named inactivators. Unlike fusion inhibitors and receptor antagonists, they can actively inactivate virions in the blood by interacting with one or more sites on the viral envelope glycoproteins. It is suggested that a number of developing protein- and peptide-based virus inactivators may be safe for use in human treatment [39]. For example, a recombinant chimera designated as DAV-EI (dual-acting virucidal entry inhibitor), consisting of cyanovirin-N (CVN) lectin and a peptide from the conserved membrane-proximal external region of HIV-1 gp41 envelope protein (MPER), could effectively inactivate HIV-1 Bal.01 pseudovirus with an EC_{50} value of 28.3 nM. Irreversible inactivation of HIV-1 virions occurred through dual interaction with gp120 and gp41. At the same time, the original CVN or MPER molecules themselves are not capable of inactivating HIV-1 virions [40].

Antiviral drugs that inhibit orthoflaviviruses

Another family of RNA-containing viruses with immense medical significance is the viruses belonging to the family *Flaviviridae* (*Riboviria*, *Orthornavirae*, *Kitrinoviricota*, *Flasuviricetes*, *Amarillovirale*, *Flaviviridae*). Today, the family *Flaviviridae* includes more than 90 species of viruses and many new, yet unclassified viruses. It is important to note that currently, orthoflaviviruses are almost globally distributed, and hundreds of millions of people encounter them annually, many become ill, and some even die. It is customary to distinguish the so-called major flavivirus infections, which are caused by the dengue, Japanese encephalitis, West Nile, yellow fever and Zika viruses.

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The hepatitis C virus (HCV), which also belongs to a separate genus within the family *Flaviviridae*, is responsible for approximately 170 million human infections. The disease often progresses in a chronic form, ending tragically, and the hepatitis C virus has quite rightly earned the unofficial title of silent killer [41]. This is the only representative of orthoflaviviruses for which officially registered highly effective drugs exist, leading to the cure of patients in most cases. These include the HCV protease inhibitors boceprevir and telaprevir [42], as well as daclatasvir, sofosbuvir and simeprevir, which target viral enzymes — NS5 polymerase or NS3 protease. They provide highly effective antiviral therapy for the overwhelming majority of patients [43]. However, in certain cases, such therapy is accompanied by adverse side effects [44]. Therefore, the search for drugs against the hepatitis C virus remains a relevant issue. Thus, a number of studies have been dedicated to substances targeting the hepatitis C virus. It has been demonstrated that harzianic acids A and B, isolated from the *Trichoderma harzianum* fungus, can affect the HCV envelope protein E1/E2 as well as the host cell CD81, inhibiting the entry of pseudoviruses into cells and exhibiting low cytotoxicity [45]. In the same HCVpp model, it was shown that the plant alkaloid berberine exhibits antiviral activity by interacting with the HCV envelope glycoproteins E1 and E2 [46]. Using pseudotyped HCV virus, it was found that LUMS1 — a modified form of the microvirin lectin, known as an HIV-1 entry inhibitor — can equally effectively inhibit the entry of HCVpp into host target cells [47]. Pangenotypic entry of HCV pseudoparticles into human Huh7 hepatoma cells was inhibited by derivatives of schisandrin acid, a triterpenoid from the *Schisandra sphenanther* fruits, preventing the fusion of the virion and the cell membrane without exhibiting significant cytotoxicity. These compounds also demonstrated strong antitumor activity against the Bel7404 and SMMC7721 cell lines [48]. Several flavonoid derivatives, in which triazole groups were combined with a pyranoflavonoid scaffold, inhibited the infection of Huh7 cells by the hepatitis C virus. Additional studies on the mechanism of action using pseudoviruses confirmed that the most effective of these compounds inhibited the virus's entry into the cell [49]. It is important to note that the hepatitis C virus is not actually cultivated in laboratory conditions on cell cultures. The successes in the development of antiviral drugs against HCV have largely been predetermined by the development and use of pseudovirus technologies for assessing the antiviral activity of candidate compounds.

Pseudotyped particles have also been created for other viruses of this family, predominantly using a lentiviral system, namely: infectious pseudotyped hepatitis C virus HCVpp carrying unmodified HCV E1 and E2 glycoproteins [50], pseudotyped Japanese encephalitis viruses [51, 52], pseudotyped dengue viruses D2(HIVluc) [53] and Zika — ZikaEnv/HIV-1 [54].

Pseudotyped viruses have been widely used to study receptor interactions between the surface proteins of orthoflaviviruses and host cells, as well as to search for and investigate the mechanism of action of antiviral drugs [55]. The list of such drugs is provided in **Table 2**.

T. Pan et al. showed that several non-steroidal anti-inflammatory drugs (NSAID), including aspirin, ibuprofen, naproxen, acetaminophen, and lornoxicam, effectively suppress the entry of pseudotyped Zika Env/HIV-1 viruses, as well as Zika virus replication in cell lines and in primary human fetal endothelial cells [54]. Interestingly, the NSAID-inhibitory effect was mediated by the effective reduction of the expression of the AXL cellular protein, a cofactor for Zika virus entry. Thus, a new mechanism of action for antiviral compounds has been described, which involves blocking the entry of the Zika virus by degrading the cofactor that facilitates the virus's entry into the cell. The authors concluded that NSAID could be used in practice to prevent Zika virus infection in pregnant women, as some NSAID, including ibuprofen and acetaminophen, are considered clinically safe.

Antiviral drugs that inhibit influenza virus entry

Influenza viruses are highly contagious respiratory pathogens of humans, belonging to the family *Orthomyxoviridae* (*Riboviria, Orthornavirae, Negarnaviricota, Polyploviricotina, Insthoviricetes, Articu-*

Table 2. Antiviral drugs against flaviviruses, obtained and/or studied using pseudovirus technology

Substance	Infection	Mechanism of action	Reference
NSAID (aspirin, ibuprofen, naproxen, acetaminophen, lornoxicam)	Zika	Reduction of cellular AXL protein expression, a cofactor for ZIKV entry	[54]
Garcianic acids A and B from *Trichoderma harzianum*	Hepatitis C*	Impact on the E1/E2 viral envelope protein, as well as CD81 of host cells	[45]
Berberine (plant alkaloid)	Hepatitis C	Interaction with glycoproteins E1 and E2	[46]
LUMS1 — a modified form of microvirin (lectin)	Hepatitis C	Interaction with glycoproteins E1 and E2	[47]
Derivatives of schisandronic acid (triterpenoid from the *Schisandra sphenanthera* fruits)	Hepatitis C	Prevent the fusion of the virion and the cell membrane	[48]

Note. * The pseudovirus is assembled based on the genome of the vesicular stomatitis virus. All other pseudoviruses are constructed based on the HIV-1 genome.

lavirales, Orthomyxoviridae). The family contains four genera of segmented RNA viruses: *Alphainfluenzavirus, Betainfluenzavirus, Deltainfluenzavirus*, and *Gammainfluenzavirus*. In fact, each genus includes one virus species: influenza A virus (*Alphainfluenzavirus influenza*), influenza B virus (*Betainfluenzavirus influenza*), influenza C virus (*Gammainfluenzavirus influenzae*), and influenza D virus (*Deltainfluenzavirus influenza*). Human diseases are mainly associated with influenza viruses A, B and C, although the family *Orthomyxoviridae* includes 5 more genera of viruses.

On the lipid envelope of influenza viruses, two main membrane glycoproteins dominate: hemagglutinin (HA) and neuraminidase (NA). The surface glycoprotein HA is responsible for the attachment of the virus and its entry into host cells through sialic acid receptors, while NA, through its enzymatic activity, ensures the release of viral progeny from the infected cell. Subtypes (serotypes) of the influenza A virus are commonly classified in combinations of 18 HA types and 11 NA types [56].

In addition to vaccination, antiviral drugs are used for the treatment and prevention of influenza A virus (IAV) infections. The search for such drugs and the study of their mechanisms of action using pseudoviral particles are the subjects of a number of studies. The list of such drugs is provided in **Table 3**.

Lentiviral pseudovirus systems have been developed for SARS-CoV, SARS-CoV-2, and avian influenza H5 [57]. However, two other platforms — the vesicular stomatitis virus genome and the mouse leukemia virus genome — are also used in research [58].

A. Antanasijevic et al., analyzing the entry of a pseudovirus based on HIV-1, found that the small molecule tert-butylhydroquinone (TBHQ) inhibits the entry of influenza virus into cells mediated by H7-type HA, as well as H3 HA. Using nuclear magnetic resonance, the authors showed that the aromatic ring of TBHQ has extensive contact with the stem loop region of H7 HA [59].

Pseudovirus-based screening allowed the identification of two super-short membrane-active lipopeptides (C12-KKWK and C12-OOWO) as effective anti-IAV agents against the influenza strains A/Puerto Rico/8/34 and A/Aichi/2/68. Inhibition of virus entry occurred through the interaction of these compounds with the HA2 subunit [60].

T.C. Hung et al. conducted a screening of a number of compounds and found that quercetin (vitamin P) inhibits the entry of pseudoviruses expressing HA of the H5N1 virus into cells [49]. Studies have shown that quercetin interacts with the HA2 subunit at an early stage of influenza infection, making it a potential candidate for the development of effective, safe, and affordable natural products for the treatment and prevention of influenza A [61].

A series of oligothiophene compounds targeting the influenza virus's HA were synthesized as specific inhibitors against the H5 subtype through a series of alkylation, azidation, amination, and amidation reactions. The inhibitory activity of these compounds was tested at the cellular level against the H5N1 influenza pseudovirus. The structural analysis of these compounds showed that the size of oligothiophene compounds is very important for their level of inhibitory activity [62].

The anti-influenza activity of pyrazolo[3,4-b]pyridinones derivatives, synthesized using an original protocol with water as a solvent, was evaluated. The protocol allowed for the synthesis of modified variants of the

Table 3. Antiviral drugs against influenza A viruses, developed and/or studied using pseudovirus technology

Substance	Influenza strain	Mechanism of action	Reference
Small molecule TBHQ	H7 A/Netherlands/219/2003, H3 A/Brisbane/10/2007	The aromatic ring of TBHQ has extensive contact with the HA stem loop region	[59]
C12-KKWK and C12-OOWO are membrane-active lipopetides	A/Puerto Rico/8/34, A/Aichi/2/68	Interaction with the HA2 subunit	[60]
Quercetin (vitamin P)	A/Anhui/1/2005A, A/Xinjiang/1/2006, A/ Hong Kong/156/1997, A/Qinghai/59/2005, A/Thailand/Kan353/2004, A/ VietNam/1194/2004	Interaction with the HA2 subunit of the influenza A (H5N1) virus, which mediates the fusion of the viral envelope with the endosomal membrane at the early stage of infection	[61]
Oligothiophene compounds	H5N1	Binding to HA	[62]
Derivatives of pyrazolo[3,4-b] pyridinediones	H5N1-A/Thailand/ Kan353/2004	Binding to HA	[63]
Pentacyclic triterpene saponins C-28	A/Duck/Guangdong/99	Binding to HA	[64]
Percolation extract of *Spatholobus suberectus Dunn*	$H5N1_{Turkey}$	Прямое связывание гликопротеинов вирусной оболочки Direct binding of viral envelope glycoproteins	[38]
Griffithsin from red algae, and its modification GL25E	H1N1: Puerto Rico/8/1934, California/07/2009, Shanghai/37T/2009, WSN/1933. H3N2: Guizhou/54/1989	Binding to HA at the stage of virus entry	[65]

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compounds within 1 hour. The results of the screening of the obtained compounds identified two derivatives that exhibited strong inhibitory activity against the pseudovirus A/Thailand/Kan353/2004. The speed and environmental friendliness of the synthesis of these derivatives open up new prospects in the field of drug development [63].

A series of modified C-28 pentacyclic triterpenoid saponins were synthesized through conjugation with amide derivatives, and their antiviral activity against the A/Duck/Guangdong/99 influenza virus in MDCK cells was evaluated. The study of mechanisms of action showed that these triterpenoids can strongly bind to the HA of the viral envelope, blocking the attachment of the H5N1 pseudovirus to target cells [64].

After the onset of the COVID-19 pandemic, drugs that inhibit the entry of influenza viruses were almost always studied in conjunction with drugs against SARS-CoV-2. Particularly close attention has been paid to natural compounds rather than chemically synthesized ones, as natural antiviral agents have been recognized as safe and effective.

For example, the aforementioned percolation extract of *Spatholobus suberectus Dunn* (SSP) is a broad-spectrum virus entry inhibitor against SARS-CoV-1/2 and other enveloped viruses. The inhibitory activity of SSP against viruses was evaluated using pseudotyped SARS-CoV-1 and 2, HIV-1ADA and HXB2, as well as H5N1.

In vivo studies have shown that even with prolonged treatment, the drug did not exhibit toxicity to the test rats compared to the control group animals. The obtained data demonstrate the potential of SSP as a candidate drug for the prevention and treatment of infections caused by enveloped viruses [37].

Griffithsin, a carbohydrate-binding protein isolated from red algae, as well as a bivalent entry inhibitor based on it (GL25E), a recombinant protein consisting of griffithsin, a 25-amino acid linker, and EK1 — a broad-spectrum coronavirus inhibitor — can effectively inhibit mono-infection with influenza A virus and SARS-CoV-2 and their co-infection by blocking HA of IAV and the spike protein of SARS-CoV-2. GL25E is more effective than griffithsin because GL25E can also interact with the HR1 domain in the S protein of SARS-CoV-2 [65].

It should be noted that the largest number of studies related to the search for antiviral drugs using pseudovirus technology is associated with the COVID-19 pandemic caused by the SARS-CoV-2 coronavirus, which has generated a real boom in virological research. A number of review publications have been published on the potential use of pseudoviruses in the study of coronavirus infections [8, 9, 65, 66]. These publications emphasize the significance of using pseudoviruses to study the interaction of SARS-CoV-2 with permissive cells, quantitatively determine neutralizing antibodies, explore new possibilities for vaccine construction, search for new antiviral drugs based on the evaluation of the activity of chemically synthesized compounds, and investigate the behavior of pseudovirus particles in the whole organism and their interaction with various organs and the immune system. The main limitations of using this technology are associated with the restricted presence of coronavirus proteins on the surface of the pseudovirus particle and the inability to use this technology to study the non-structural proteins of SARS-CoV-2. Overall, the number of publications on pseudoviruses and SARS-CoV-2 is simply enormous and requires separate consideration.

Conclusion

An analysis was conducted on the use of pseudoviruses for the development of new diagnostic, preventive, and therapeutic tools for a number of serious socially significant infectious diseases caused by RNA-containing viruses, based on data published in recent years. Currently, technologies using pseudoviruses are widely and successfully employed for research on HIV-1, hepatitis C virus, tick-borne encephalitis virus, avian influenza viruses, and SARS-CoV-2, as well as for particularly dangerous infection viruses, such as Marburg and Ebola viruses [67, 68].

The success of using pseudoviruses is determined by the fundamentally new capabilities of this technology:

- allows conducting research on the initial stage of virus entry into the cell;
- allows conducting research under conditions that ensure a high level of biosafety, especially when working with pseudoviruses that model highly pathogenic viral agents;
- principally simplifies the conduct of research and makes it possible, especially for poorly cultivable or uncultivated viruses;
- expands the experimental capabilities of researchers;
- successfully combines with modern methods of synthetic biology and bioinformatics.

The collection of presented data illustrates the fundamentally new contribution of pseudovirus technology to the search and creation of a new generation of drugs to combat serious socially significant diseases caused by RNA-containing viruses.

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Review
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Bioconjugation as a promising method for vaccine development

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Abstract

Introduction. Bioconjugation, or protein glycan coupling technology, PGCT, is a method for creating carbohydrate-protein composites based on the ability of certain bacteria to perform eukaryotic-type glycosylation. This method allows for the production of glycoproteins directly in the cells of producer bacteria, most often *Escherichia coli*, bypassing the stage of chemical conjugation. This significantly simplifies the creation and production of conjugated vaccines, consisting of polysaccharide antigens combined with a protein carrier that performs the functions of a T-cell antigen and an adjuvant.

The aim of the review is to analyze and summarize current data on both the bioconjugation method itself and the underlying biochemical processes, as well as on the vaccines being developed using this method.

The preparation of the review involved studies presented in the PubMed, Scopus, Google Scholar, eLIBRARY.RU databases as of February 2025. The following keywords were used for the search: bioconjugation, vaccines, PGCT, conjugated vaccines, bacterial glycosylation.

An analysis of literature sources dedicated to the study of bacterial N-glycosylation, on the basis of which the bioconjugation technology was developed, as well as similar processes occurring in certain bacterial species, was conducted. Reports on the development of new vaccines and the improvement of existing vaccines against the most relevant pathogens have been analyzed. At present, vaccination appears to be the most effective way to combat infectious diseases, including efforts to counter the spread of antibiotic-resistant microorganisms. The diversity of pathogens encountered by the human population compels the search for multiple approaches of creating effective and safe vaccines. Simplifying and reducing the cost of producing new drugs allows for a more confident response to the threat of new epidemics. Bioconjugation helps create new vaccines and improve existing vaccines, although there are certain limitations**.**

Conclusion. Modern vaccine production is characterized by a variety of approaches united by a single goal — to effectively counter the threats of new epidemics. Bioconjugation is one of the new, yet quite promising methods through which several vaccine candidates are already being developed. The analysis of the current state of these projects may be useful in choosing an approach for developing subsequent preventive immunological drugs.

Keywords: *review, vaccines, bacterial glycosylation, bioconjugation, recombinant proteins*

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Биоконъюгация как перспективный метод создания вакцин

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Аннотация

Введение. Биоконъюгация, или технология рекомбинантного бактериального гликозилирования (protein glycan coupling technology, PGCT), — это метод создания углеводно-белковых композитов, основанный на способности некоторых бактерий осуществлять гликозилирование по типу эукариот и позволяющий получать гликопротеины непосредственно в клетках бактерий-продуцентов, чаще всего *Escherichia coli*, минуя стадию химической конъюгации. Это значительно упрощает создание и производство конъюгированных вакцин, состоящих из полисахаридных антигенов, объединённых с белковым носителем, выполняющим функции Т-клеточного антигена и адъюванта.

Цель обзора: проанализировать и обобщить актуальные данные как о самом методе биоконъюгации, так и о биохимических процессах, лежащих в его основе, а также о разрабатываемых с его использованием вакцинах.

При подготовке обзора были рассмотрены работы, представленные в базах PubMed, Scopus, Google Scholar, eLIBRARY.RU по состоянию на февраль 2025 г. Для поиска использовали следующие ключевые слова: bioconjugation, vaccines, PGCT, биоконъюгация, конъюгированные вакцины, бактериальное гликозилирование.

Проведён анализ источников литературы, посвящённых изучению бактериального N-гликозилирования, на базе которого была создана технология биоконъюгации, а также сходных с ним процессов, протекающих в отдельных видах бактерий. Проанализированы сообщения о разработке новых и усовершенствовании уже имеющихся вакцин против наиболее актуальных патогенов. В настоящий момент вакцинация представляется наиболее эффективным способом борьбы с инфекционными заболеваниями, включая также противодействие распространению антибиотикорезистентных микроорганизмов. Разнообразие патогенов, с которыми сталкивается человечество, вынуждает искать множественные подходы для создания эффективных и безопасных вакцин. Упрощение и снижение себестоимости производства новых препаратов даёт возможность более уверенно противостоять угрозе новых эпидемий. Биоконъюгация помогает создавать новые вакцины и совершенствовать уже имеющиеся, хотя и обладает определёнными ограничениями.

Заключение. Современное производство вакцин характеризуется разнообразием подходов, объединённых одной целью — эффективно противостоять угрозам новых эпидемий. Биоконъюгация — один из новых, но довольно многообещающих методов, с помощью которого уже разрабатывается несколько вакцин-кандидатов. Анализ текущего состояния этих проектов может быть полезен при выборе подхода для создания последующих профилактических иммунопрепаратов.

Ключевые слова: *обзор, вакцины, бактериальное гликозилирование, биоконъюгация, рекомбинантные белки*

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Introduction

In November 2024, the World Health Organization (WHO) updated the list of priority endemic pathogens for which there is the greatest need for vaccines [1], noting that vaccination not only reduces morbidity but also decreases antibiotic use, thereby lowering mortality caused by antibiotic-resistant strains. According to WHO estimates, vaccination against 23 pathogens could reduce the need for antibiotics by 22% [2]. Moreover, the widespread use of existing vaccines against pneumococcus, *Haemophilus influenzae* type b, and typhoid fever could potentially prevent up to 106,000

deaths annually caused by the spread of antimicrobial resistance. The development and global implementation of new vaccines against *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* could potentially prevent more than 500,000 deaths annually caused by antimicrobial resistance. To create the most effective vaccines, a variety of approaches are currently being used. One of them is bioconjugation or protein glycan coupling technology (PGCT).

The aim of this review is to analyze and summarize current data on both the bioconjugation method itself and the biochemical processes underlying it, as well as on the vaccines being developed using this method.

In preparing the review, an analysis was conducted of both English- and Russian-language literature available in the scientific databases PubMed, Scopus, Google Scholar, and eLIBRARY.RU as of February 2025. The following keywords were used for the search: bioconjugation, vaccines, PGCT, bioconjugation, conjugated vaccines, bacterial glycosylation. At the first stage, when searching for "bioconjugation" from 1968 to 2025, more than 6000 sources were found, which were reduced to 250 by combining queries. Initially, studies from 2010 to 2025 were selected, reducing the number to 178, after which a number of relevant articles without time restrictions were added for a more comprehensive coverage of the research issue. Due to the article's length limitation, 59 of the most relevant sources were selected. Also, studies for which it was impossible to obtain the full text of the article, sources not in English, as well as those Russian-language articles in which the issue was mentioned but not discussed in detail, limiting themselves to references to already used foreign sources in the review, were excluded from the selection.

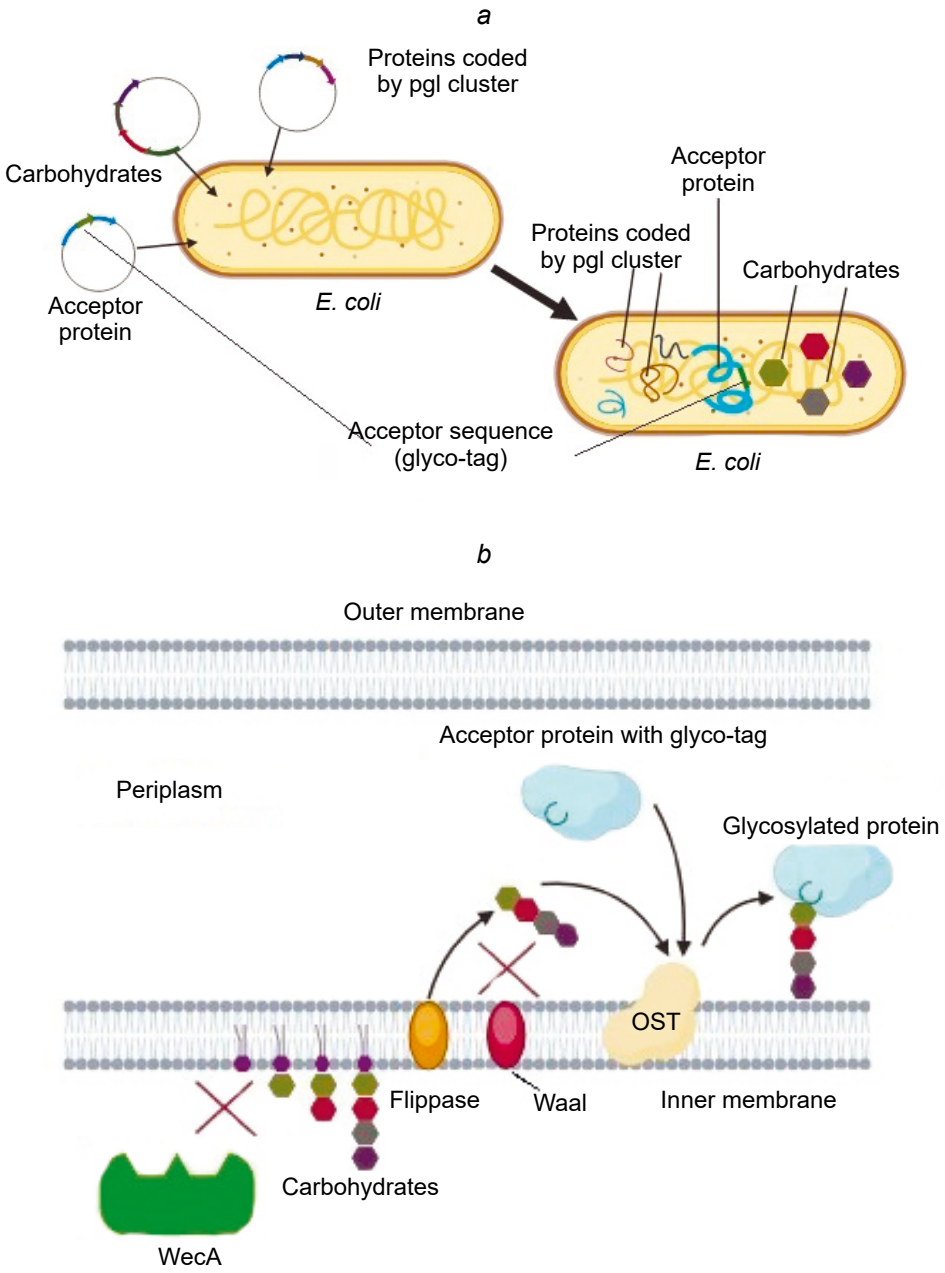
Key features of bioconjugation

PGCT was created based on the ability of the microorganism *Campylobacter jejuni* to perform N-glycosylation of proteins using the oligosaccharyltransferase (OST) PglB, which is expressed along with other enzymes in a cluster called pgl (protein glycosylation), as described in 1999 [3]. PglB is a membrane enzyme with an active site facing the periplasmic space. It is capable of attaching an oligosaccharide, primarily composed of N-acetylgalactosamine (GalNac) monomers, to the asparagine residue located at the center of the so-called acceptor sequence [4]. The acceptor sequence of PglB is as follows: Asp/Glu — Y — Asn — X — Ser/Thr, where X and Y are any amino acids except proline [5]. The attachment of the oligosaccharide occurs through the amide group of asparagine and is classified as N-glycosylation. The pgl cluster has been successfully cloned into *E. coli* and has demonstrated the ability to express the entire set of enzymes necessary for glycosylation, as well as perform the glycosylation itself when proteins with the required acceptor sequence are present [6]. Thus, it became possible to obtain glycosylated proteins directly in *E. coli*. This process was named bioconjugation, or PGCT technology (**Figure**).

Historically, in the production of polysaccharide-based vaccines, carbohydrates are chemically covalently attached to a carrier protein, which serves the functions of a T-cell antigen and partially an adjuvant. The discovery of bacterial N-glycosylation and the possibility of its functional transfer to *E. coli* allowed for the development of products in which both the production of antigens and their conjugation occur directly in the producing organism, simplifying and reducing the cost of the process.

The first attempts to create a vaccine using PGCT technologies appeared in 2010 [7]. J. Ihssen et al. published a report on the successful production of conjugates in *E. coli*, consisting of the O-antigen of *Shigella dysenteriae* serotype 1 and the AcrA proteins of *C. jejuni* and exotoxin A of *Pseudomonas aeruginosa* (EPA), respectively. After this report, news about the development of new vaccines against various pathogens based on PGCT began to arrive regularly. However, difficulties immediately arose, primarily due to the fact that PglB is capable of transferring only oligosaccharides with a reducing terminal residue, catalyzing the formation of a bond through the acetamido group at the 2-position, which sharply limits the number of carbohydrate antigens that can be used. To overcome these limitations, researchers began using O-glycosylating bacterial OST, which were found in species such as *P. aeruginosa*, *Neisseria meningitides*, *Francisella tularensis* and *Acinetobacter baylyi*.

In the case of *P. aeruginosa*, the function of OST is performed by the PilO enzyme, which transfers glycans to the PilA protein (type IV pilin) [8]. *N. meningitides* expresses the PglL protein, encoded by the pglL gene, which also glycosylates pili [9]. *F. tularensis* contains the enzyme PglA, which attaches pentasaccharides to the PilA protein [10]. The PglS enzyme from *A. baylyi* is an O-glycosyltransferase that transfers carbohydrate residues to the pilin-like protein ComP [11]. All the listed proteins, when cloned in *E. coli*, demonstrated the ability to glycosylate their native substrates [12]. In this case, PilO transferred only short oligosaccharides, whereas PglL performed glycosylation with long oligosaccharides and, additionally, interacted with glycans that were inaccessible to PglB, such as the O4 O-antigen of *Salmonella typhimurium*. Regarding PglS, it was found that it is capable of transferring oligosaccharides with glucose as the reducing terminal sugar, which distinguishes it favorably from other bacterial OST [13]. Using these enzymes, researchers were able to develop and obtain bioconjugates capable of eliciting immune responses in both laboratory animals and humans. At present, potential bioconjugate vaccines against Shigella, pathogenic *E. coli*, *Klebsiella*, *Streptococcus pneumoniae*, *Brucella, Staphylococcus aureus* and other



General scheme of PGCT in *E. coli* cells [18, with added changes].

a — the stage of transforming bacteria with plasmids containing nucleotide sequences that encode, respectively, a carrier protein with an acceptor sequence specific to the used OST, a set of carbohydrates necessary for its glycosylation, and a cluster containing the enzymes required for glycosylation; *b* — the process of glycosylation itself, occurring on the inner membrane of *E. coli* cells modified for more efficient bioconjugate synthesis.

WaaL is the O-antigen ligase of *E. coli*, competing with recombinant OST for oligosaccharides; WecA is the enzyme that catalyzes the biosynthesis of native glycans in the producing bacterium; flippase is the enzyme that transfers the carbohydrate sequence to the periplasm.

pathogens posing the greatest threat to public health are at various stages of development [2].

Bioconjugate vaccines against Shigella

Shigellosis or bacterial dysentery is caused by microbes of the genus *Shigella*. These are gram-negative bacteria from the family *Enterobacteriaceae* that penetrate the gastrointestinal tract, infect the mucous membrane of the large intestine, and cause inflammation. Shigellosis is one of the leading causes of diarrhea-related deaths worldwide. Children under the age of 5 in low- and middle-income countries suffer the most [14]. Currently, four species of Shigella are known: *S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii*. The greatest threat is posed by the species *S. flexneri*. There are 15 known serotypes of *S. flexneri*, the most common of which is *S. flexneri* 2a, followed by *S. flexneri* 3a and *S. flexneri* 6. *S. sonnei* is the prevalent species of *Shigella* in industrialized countries, and one serotype is known for it. Despite the large number of develop-

ments that have reached the clinical trial stage [15] (**Table**), there is currently no licensed international vaccine against shigellosis.

The first bioconjugated vaccine tested in humans was a candidate vaccine against *S. dysenteriae* [16]. In its development, the O-antigen polysaccharide of *S. dysenteriae* type O1 was bioconjugated in *E. coli* with a recombinant version of EPA and the PglB cluster, named GVXN SD133, and underwent Phase I clinical trials [17]. The results showed that regardless of the method of administration, the drug was well tolerated and had an acceptable level of safety. In the blood of the vaccinated, a statistically significant increase in the levels of IgG and IgA class antibodies against the O1 polysaccharide was detected [16].

Then, another monovalent vaccine against Shigella based on the O-antigen of *S. flexneri* 2a was developed, also based on PglB. The same rEPA was chosen as the protein carrier. The bioconjugate was named Flexyn 2a. The safety and immunogenicity of this vaccine prototype have been confirmed. Similarly, to the results obtained in the study of the vaccine against *S. dysenteriae*, immunization with the Flexyn 2a drug revealed a significant increase in the titers of IgG and IgA antibodies against LPS of *S. flexneri* 2a [18]. A randomized double-blind placebo-controlled phase 2b study [19] demonstrated an adequate level of safety and immunogenicity of the vaccine. The efficacy of the Flexyn 2a bioconjugate was further confirmed by other methods, including the assessment of the severity of shigellosis. It was shown that this indicator was lower in the vaccinated individuals than in the patients receiving the placebo [20].

Promising results demonstrated during the development and testing of Flexyn 2a contributed to the development of a polyvalent vaccine. S4V is a quadri-

Bioconjugated vaccines against various pathogens currently under development

Pathogen	Drug name/Bioconjugate characteristics	Stage	Reference
Shigella spp.	GVXN SD133	Phase I clinical trials	[17]
	Flexyn 2a	Phase 2b clinical trials	[19]
	S4V	Phase 1/2 clinical trials	NCT04056117 — ClinicalTrials.gov
Pathogenic *E. coli* (*E. coli* O 157)	Conjugate of O-antigen and *E. coli* O157 MBP, OST — PglB	Preclinical trials on mice	[23]
	Conjugate of O-antigen and *Citrobacter sedlakii* CmeA protein NRC6070, OST — PglB	Laboratory experiments	[25]
Extraintestinal pathogenic *E. coli*	ExPEC9V	Phase III clinical trials	NCT04899336 — ClinicalTrials.gov
	ExPEC10V	Phase I and II clinical trials	NCT04306302, NCT03819049 — ClinicalTrials.gov
	Conjugate of O25B-antigen ExPEC and EPA, OST — PglB	Laboratory experiments	[32]
Hypervirulent type *K. pneumoniae* (hvKp)	Conjugate of capsular polysaccharides of serotypes K1 and K2 with the EPA-ComP protein, OST — PglS	Preclinical trials in mice	[34]
K. pneumoniae	Conjugate of O-antigens of lipopolysaccharide and protein EPA, OST — PglS	Preclinical trials in mice	[36]
K. pneumoniae O1	KPO1-VLP	Preclinical trials in mice	[37]
S. pneumonia	Conjugate of serotype 4 capsular polysaccharide and AcrA protein, OST — PglB	Preclinical trials in mice	[39]
	Conjugates of the ST4 capsular polysaccharide and the NanA, Sp-148, PiuA proteins, OST – PglB	Preclinical trials in mice	[43]
	CPS8-EPA^{iGTcc}	Preclinical trials in mice	[44]
S. agalactiae	Conjugate of polysaccharides of serotypes Ia, Ib and III with the EPA-ComP protein, OST — PglS	Preclinical trials in mice	[47]
B. abortus	Conjugate of *Yersinia enterocolitica* O-polysaccharides and cholera toxin B, OST — PglL	Preclinical trials in mice	[52]
	Conjugate of *Brucella* O-polysaccharides and Nano-B5 nanoparticles, OST — PglL	Preclinical trials in mice	[54]
S. aureus	CP5-EPA, CP8-EPA and CP5-Hla	Preclinical trials in mice	[56]
F. tularensis	Conjugate of the O-antigen of *F. tularensis* and EPA	Preclinical trials in mice	[58]
	Conjugate of the O-antigen of *F. tularensis* and the CmeA protein of *Citrobacter sedlakii* NRC6070, OST — PglB	Laboratory experiments	[25]

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valent bioconjugated vaccine that contains O-antigens of *S. flexneri* serotypes 2a, 3a, 6, as well as *S. sonnei*, conjugated to the carrier protein EPA. Currently, a double-blind S4V study is being conducted in Kenya to determine the appropriate dose and age group (adults, children, infants). The data collected during this study will be an important step in the development of a vaccine against *Shigella* [21].

Bioconjugated vaccines against pathogenic strains of E. coli

Pathogenic strains of *E. coli* are divided into two groups: extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC). ExPEC strains are primarily associated with neonatal meningitis and urinary tract infections in adults (UTIs). InPEC strains cause various diarrheal diseases and are divided into 6 pathotypes, including enter hemorrhagic *E. coli* strains. One of the most common representatives of the EHEC group is enterohemorrhagic Escherichia coli O157:H7 (*E. coli* O157), which causes diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome [22]. The need for vaccines to prevent *E. coli* O157 is very high. Their development has been ongoing for quite some time, and several drugs are undergoing preclinical and clinical trials, including a bioconjugated vaccine [23]. As a carrier protein, the authors chose the maltose-binding protein because recent studies have shown that it is a TLR4 agonist and induces the activation of the NF-κB signaling pathway, as well as the secretion of a number of pro-inflammatory cytokines [24]. Conjugation was carried out using PglB, attaching the O-antigen of *E. coli* O157 to the protein, with the producer organism being the *E. coli* W3110 strain. The resulting bioconjugate induced the activation of both the humoral and cellular immune responses [23].

Another prototype of a bioconjugated vaccine against *E. coli* O157 was created using the experimental MAGIC technology (Mobile-element Assisted Glycoconjugation by Insertion on Chromosome) [25]. The essence of the method lies in the use of mobile genetic elements, specifically the tn5 transposon, to integrate constructed genetic sequences into the *E. coli* chromosome. The developers of MAGIC claim that such a construct significantly alleviates the metabolic burden and directly promotes the increase in producer biomass and the yield of the bioconjugate. To achieve this result, they used the tn5 transposon, into which segments encoding PglB, the *C. jejuni* AcrA carrier protein, and enzymes involved in polysaccharide biosynthesis [26] were integrated into the nucleotide sequence. The bioconjugated prototype of the MAGIC vaccine was obtained in the non-pathogenic bacterium *Citrobacter sedlakii* NRC6070. The carrier protein used to create the preparation was CmeA 6xHis, the polysaccharide component was the O-antigen of *C. sedlakii*, which is similar to that of *E. coli* O157, and PglB was used as the OST. In terms of its biochemical parameters, the bioconjugate met the stated requirements. Unfortunately, there is currently no data on any clinical trials of the obtained drug.

Extraintestinal strains of pathogenic *E. coli* (ExPEC) are also quite dangerous, as they are capable of causing various types of diseases. ExPEC strains are classified into three main pathotypes: uropathogenic *E. coli* (UPEC), sepsis-causing *E. coli* (SEPEC), and *E. coli* associated with neonatal meningitis [27]. Unfortunately, UTIs caused by ExPEC are extremely difficult to treat. The creation of effective vaccines that prevent such developments is an extremely important task. To address this, along with other approaches, PGCT methods were also applied. The study began with the creation of a quadrivalent prototype, which included 4 EPA-conjugated variants of the O-antigen. High tolerability of the prototype and a significant increase in IgG antibody levels against all antigens, as well as a reduction in the number of reported UTI cases among trial participants, have been demonstrated [28, 29]. Based on the 4-valent prototype, a 9-valent vaccine ExPEC9V has been developed, containing a conjugated polysaccharide and currently undergoing phase III clinical trials (NCT04899336).

The safety and immunogenicity of the 10-valent ExPEC10V vaccine among elderly individuals aged 60–85 years are still being studied (Phases I and II, NCT04306302, NCT03819049). Like the quadrivalent prototype, this drug is well tolerated and induces the production of antigen-specific antibodies in the majority of participants, despite their advanced age [30].

A similar approach was used in the development of the O25B antigen ExPEC vaccine prototype. Although the O-antigen of *E. coli* has more than 180 serotypes, a significant number of isolates obtained from UTI carriers belong to the O25B serotype [31]. Therefore, a group of researchers attempted to create a vaccine based specifically on this antigen. The O-antigen cluster was integrated into the *E. coli* W3110 genome, after which the expressed polysaccharide was enzymatically conjugated with the EPA enzyme PglB. Detailed characterization of the O25B-EPA conjugate using physicochemical methods, including nuclear magnetic resonance and gas chromatography-mass spectrometry, confirmed its correspondence to the O25B structure, thereby opening up the possibility for the development of a polyvalent conjugate vaccine against ExPEC [32].

Bioconjugated vaccines against *Klebsiella pneumoniae*

The Gram-negative bacterium *Klebsiella pneumoniae* is the second most common opportunistic pathogen after *E. coli*. It causes neonatal sepsis, UTI and nosocomial pneumonias that are poorly treatable due to antimicrobial resistance — the bacteria acquire resistance factors such as extended-spectrum β-lactamases

and *K. pneumoniae* carbapenemases. The WHO has assigned the highest danger levels to isolates containing these factors [33], confirming the urgent need for the development of an effective and safe vaccine.

Using PGCT technology, several vaccine formulations are being developed. M.F. Feldman and co-authors focused on developing a vaccine against hypervirulent *K. pneumoniae* (hvKp) because this particular strain of the pathogen is the most dangerous. If other serotypes generally cause diseases in hospitalized patients, the elderly, infants, or people with various types of immunodeficiencies, hvKp pose a threat even to healthy individuals [34]. The mechanisms of hypervirulence are not fully understood, but it is suggested that the main reason is the excess of capsular polysaccharide, which hinders the elimination of the pathogen from the body. As the main O-antigen synthase, the researchers chose PglS from *A. baylyi*, and as the carrier protein, they used EPA fused with the ComP protein. As the carbohydrate component, the capsular polysaccharides of the most common serotypes K1 and K2 were used, the synthesis clusters of which were cloned into *E. coli* producer cells with partially blocked natural glycosyltransferases. The obtained glycoproteins, when administered to mice, demonstrated the ability to induce the synthesis of protective IgG1 antibodies, significantly increasing the survival rate of mice upon subsequent infection.

Another vaccine is being developed against the classical serotypes of *K. pneumonia*. As a carbohydrate component, O-antigens of bacterial lipopolysaccharides are used. Unlike capsular polysaccharides, only 11 serotypes of O-antigens expressed by *K. pneumoniae* are currently known [35]. Based on the 7 most common O-antigen serotypes, a heptavalent bioconjugate vaccine was constructed. The PglS enzyme was chosen as the OST, and the carrier protein was recombinant EPA with an inserted acceptor sequence for PglS. The *E. coli* CLM24 strain was used as the producer. After isolation and purification, glycoproteins of all 7 types were used for mouse immunization, which was accompanied by the production of a high level of IgG antibodies to all glycoproteins. However, the bactericidal activity of the antibodies against various strains of *K. pneumoniae* was low, indicating the need for further development of the vaccine. In this regard, the authors suggest incorporating capsular antigens into the vaccine composition [36].

Another vaccine against *K. pneumoniae* using PGCT is being developed based on PglL. As an acceptor protein, a universal recombinant protein SpyCatcher4573 and a specially modified strain of *E. coli*, in which both key components SC4573 and PglL are integrated into the genome, were used. Glycoproteins obtained in this way can spontaneously bind to protein nanocarriers *in vitro* using the SpyTag system, forming conjugated nanovaccines. To enhance the efficiency of glycoprotein expression, the yfdGHI gene cluster was removed. The obtained conjugated nanovaccine against *K. pneumoniae* O1 (KPO1-VLP) demonstrated its effectiveness in experiments, where high antibody titers and 100% protection against infection with a virulent strain were observed after three immunizations [37].

Bioconjugated vaccines against *Streptococcus pneumoniae*

S. pneumoniae, pneumococcus, is one of the most common and harmful causative agents of bacterial pneumonia, meningitis, and sepsis. Despite the availability of vaccines, *S. pneumoniae* still causes over 1 million deaths annually, primarily among children under 5 years old in low- and middle-income countries [38]. Since most of the capsular polysaccharides of *S. pneumoniae* contain a terminal sugar that is not transferred by PglB, the initial attempts to create a bioconjugated vaccine focused on serotype 4, where the terminal residue was the recognizable GalNac. The acceptor chosen was the native *C. jejuni* AcrA protein, and PglB, cloned into the chromosome of *E. coli* W3110, was used as the OST. The obtained preparation protected mice upon subsequent infection with *S. pneumoniae* serotype 4 [39].

The next variant of the bioconjugated vaccine was developed based on native *S. pneumoniae* proteins. It was assumed that this would create heterologous protection against vaccine-uncovered serotypes and enhance mucosal immune protection by stimulating Th17 activation. The authors tested the efficacy of a trivalent bioconjugate in mouse models, which included the ST4 capsular polysaccharide and three protein antigens of *S. pneumoniae*: the N-terminal fragment NanA, a virulence factor that promotes growth and survival in the nasopharyngeal tract, invasion of brain endothelial cells [40], the Th17-stimulating antigen Sp0148 [41], and the ABC transporter lipoprotein PiuA [42]. The bioconjugates obtained using PglB in *E. coli* induced the synthesis of anti-capsular antibodies in mice at a level comparable to existing vaccines, and also elicited strong responses to protein antigens, which extended to other, heterologous serotypes. The authors also noted that the expression of several serotypes of capsular polysaccharides in *E. coli* opens up new possibilities for designing vaccines against *S. pneumoniae*. For example, glycosylated outer membrane vesicles (glyOMV) can be used as a platform [43].

Another prototype, this time polyvalent, was created using the PglS enzyme from *A. baylyi* as the main OST, capable of transferring the glucose terminal residue. As a protein acceptor, the natural substrate of PglS, the pilin-like protein ComP, was used, which in *E. coli* was glycosylated with the capsule polysaccharides *S. pneumoniae* CPS8, CPS9V and CPS14. The obtained vaccine showed immunogenicity in preliminary tests comparable to the immunogenicity of the Prevnar13 vaccine. Moreover, the sera of mice immunized with the obtained vaccine exhibited bactericidal activity against

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S. pneumoniae serotypes 14 and 8. Developing the idea further, the authors constructed a bioconjugate based on the protein carrier EPA, modifying its C-terminus by attaching to it an acceptor sequence of 23 amino acids from the ComP protein, and the pneumococcal polysaccharide CPS8. The obtained bioconjugate induced the active formation of IgG antibodies in mice and exhibited protective effects [13]. In 2022, it was shown that the bioconjugate obtained by the authors, named CPS8-EPAiGTcc, possesses high immunogenicity, induces the formation of IgM and IgG antibodies specific to serotype CPS8 in mice, and provides protection against infection with *S. pneumoniae* serotype 8 [44].

Bioconjugated vaccines against other types of streptococci

Bioconjugated vaccines against pathogenic streptococci are also being developed. Group B Streptococcus (GBS, *Streptococcus agalactiae*, β-hemolytic streptococcus B) is a gram-positive opportunistic bacterium that most often colonizes the lower parts of the gastrointestinal tract and the urogenital system. Approximately 10–35% of women are infected with GBS, which can lead to various acute diseases in pregnant and postpartum women, as well as to stillbirth [45]. GBS can also be transmitted to the newborn. It usually manifests as Group B Streptococcal disease and can cause meningitis, sepsis and pneumonia. Moreover, recent studies have shown that GBS is also responsible for a significant number of illnesses in adults, especially those over 65 years old [46]. All of this makes the development of a vaccine against GBS extremely necessary. In this regard, a vaccine based on PGCT was developed. The characterization of the trivalent bioconjugate vaccine targeting the three most clinically prevalent serotypes of GBS: Ia, Ib, III, is included in a study by J.A. Duke et al. [47]. The authors introduced loci necessary for the expression of the PglS protein from *A. baylyi* into *E. coli*, which allowed for the glycosylation of the constructed EPA-based carrier protein and the ComP protein with sialic acid residues according to the GBS serotypes Ia, Ib, and III. Further immunization of mice with the obtained vaccine showed that the trivalent bioconjugated vaccine against *S. pneumoniae* induces the production of IgG antibodies specific to the involved serotypes, which possess neutralizing ability. However, the effectiveness of antibodies against different serotypes used in the creation of the vaccine varied, and the authors suggested that this effect could be eliminated by altering the degree of glycosylation of the carrier protein.

PGCT technologies can also be applied to create a vaccine against group A streptococcus (*Streptococcus pyogenes* or group A strep). *S. pyogenes* is an extremely common pathogen, causing a wide range of diseases from acute pharyngitis and impetigo to scarlet fever and invasive diseases such as toxic shock syndrome or necrotizing fasciitis. They lead to the development of secondary autoimmune diseases, such as rheumatic heart disease [48]. Moreover, humans are the only natural hosts of *S. pyogenes*, therefore, blocking the transmission of this pathogen could lead to its complete elimination. Streptococcus A, like *S. pneumoniae*, has high antigenic heterogeneity. Serotypes are determined by differences in the main virulence factor, protein M. Due to such heterogeneity in the surface proteins of *S. pyogenes*, researchers have focused on developing conjugated vaccines based on the external polysaccharides of the pathogen, particularly group A polysaccharide. However, R. Di Benedetto et al. showed that for greater effectiveness of the future vaccine, it is necessary to preserve the protein epitopes of the carriers, as random conjugation did not affect the synthesis of IgG to the group A polysaccharide component but significantly reduced the response to the protein component [49]. As a result of the random conjugation of group A polysaccharide with three *S. pyogenes* proteins (SLO, SpyAD and SpyCEP), conjugates were obtained, and immunization with them led to the production of antibodies that did not block the activity of one of the proteins used for conjugation — SpyCEP. It retained the ability to cleave interleukin-8. Apparently, to create an effective vaccine based on the native proteins of *S. pyogenes* and its own group A polysaccharide, it will be necessary to ensure an extremely high precision in attaching the polysaccharide to specific sites on the proteins, which can be quite effectively achieved using PGCT [50].

Other bioconjugate vaccines

PGCT technologies are used in the development of a vaccine against *B. abortus* [51]. This pathogen, although primarily a cause of diseases in livestock, nevertheless poses a threat to humans as well. There is currently no licensed vaccine that protects against *B. abortus* infection. There are attenuated vaccines used to protect large (S19 and RB51) and small (Rev1) cattle [52]. However, these vaccines are pathogenic to humans, have residual toxicity for animals, and do not protect against all known species of the pathogen. Moreover, working with *Brucella* cultures requires high-level biosafety equipment due to the risk of aerosol transmission. To avoid these difficulties, *Y. enterocolitica*, a less dangerous opportunistic pathogen, is often used for the synthesis of glycosylated glycoproteins similar to *B. abortus*, as the O-polysaccharides of *Brucella* and *Y. enterocolitica* are similar [53]. At present, prototypes of vaccines based on cholera toxin B as a protein carrier and O-polysaccharides of *Y. enterocolitica* synthesized in genetically modified *E. coli* [52], as well as those based on Nano-B5 nanoparticles as a platform and O-polysaccharides of *Brucella* [54], are at a high level of readiness. The producer in the latter case is *Y. enterocolitica*. Both vaccines used an O-glycosylation system with the central OST PglL

from *N. meningitides*. In both cases, the researchers report successful application of the obtained prototypes in preclinical studies on mice. When administered to animals, both increased antibody production and activation of cellular immunity were observed. Moreover, both prototypes demonstrated a strong protective effect of immunization followed by infection in mice, and in the case of the nanovaccine, even against several species of *Brucella*. Further clinical studies will obviously demonstrate the applicability of the obtained drugs for human immunization.

Staphylococcus aureus is responsible for numerous human diseases, including endocarditis, pneumonia and wound infections. The methicillin-resistant *S. aureus* (so-called MRSA strains) [55] poses a particular danger. In this regard, there is an urgent need for effective vaccination against staphylococcal infection. In a study by M. Wacker et al., the results of testing 3 conjugates obtained using PGCT technologies on mice were presented. By the names of the components they contain, they are called CP5-ETA, CP8-ETA and CP5-Hla, where CP5 and CP8 are capsular polysaccharides of *S. aureus* serotypes 5 and 8, respectively, ETA is exotoxin A of *P. aeruginosa*, and Hla is α-toxin of *S. aureus*. In the study, PglB from *C. jejuni* was used. Bioconjugates were synthesized in *E. coli* and then administered to mice. All three prototypes induced a high level of antibody production. When evaluating the protective efficacy of the drugs, the best results were shown by the conjugate CP5-Hla; the administration of CP5-Epa and CP8-Epa significantly reduced bacteremia; the bioconjugated vaccine CP5-Hla protected against both bacteria and fatal pneumonia.

The PGCT technology was applied to create vaccine prototypes against *Francisella tularensis*, an intracellular pathogen causing tularemia — a potentially fatal disease. For humans, the two most dangerous subspecies are *F. tularensis tularensis* (type A) and *F. tularensis holarctica* (type B) [57]. The authors used PglB to obtain a bioconjugate in *E. coli* consisting of the O-antigen of *F. tularensis* and EPA, and tested it on a mouse model. The obtained recombinant bioconjugate exhibited high yield, stimulated the production of specific antibodies, and provided protection against subsequent infection with the virulent wild-type strain *F. tularensis* subsp. *holarctica* [58]. The authors subsequently modified the EPA carrier by adding 8 additional acceptor sequences to increase its glycosylation degree [59]. The new bioconjugate actually stimulated the formation of specific antibodies more effectively, protecting rats from developing the disease when infected with *F. tularensis*. The researchers plan to continue working on the presented vaccine prototype, intending to replace the carrier protein EPA with native protein antigens of *F. tularensis*.

Another attempt to create a new vaccine against *F. tularensis* was associated with the use of the already mentioned experimental technology MAGIC [25]. PglB was used as the OST, the carrier protein was periplasmic Cmea from *C. jejuni*, equipped with a 6His tag for ease of purification, and the polysaccharide was the O-antigen of *F. tularensis*. The producing organism was *E. coli*, and all components were integrated into the bacterial chromosome. The study demonstrated the effectiveness of the MAGIC technology in obtaining highly immunogenic bioconjugates.

Conclusion

Thus, bioconjugation, like other modern technologies, is actively used in the development of new vaccines and the improvement of existing vaccines. Despite the current limitations, this method can be used to create drugs that prevent infectious diseases, thereby reducing the spread of antibiotic-resistant microorganisms.

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К 80-летию со дня рождения Олега Ивановича Киселева

Олег Иванович Киселев родился 5 сентября 1945 г. в Магнитогорске в семье журналиста Ивана Яковлевича Киселева и преподавателя русского языка и литературы Галины Григорьевны Киселевой. В 1962 г. окончил школу в Челябинске, а в 1968 г. — с отличием Первый Ленинградский медицинский институт им. И.П. Павлова, получив официальную рекомендацию для поступления в аспирантуру. С институтской скамьи Олег Иванович увлекался микробиологией и биохимией. Он поступил в аспирантуру в лабораторию биохимической генетики Института экспериментальной медицины АМН СССР и уже в 1971 г. успешно защитил диссертацию на соискание учёной степени кандидата медицинских наук по специальности «Биохимия». Продолжив работать в Институте экспериментальной медицины, в 1982 г. Олег Иванович защитил докторскую диссертацию «Белоксинтезирующие структуры митохондрий и топография биосинтеза митохондриальных белков» по специальности «Биологическая химия».

С 1983 г. он продолжил свой научный путь в учреждениях Главного управления микробиологической промышленности при Совете Министров СССР (Главмикробиопром) и Министерства медицинской и микробиологической промышленности СССР, став руководителем Отделения генной инженерии при Главмикробиопроме. Этот период творческого пути О.И. Киселева был сфокусирован на вопросах генной инженерии и промышленной биотехнологии. Основные работы были посвящены клонированию и экспрессии генов интерферонов и цитокинов (интерлейкин-2).

В 1988 г. Олег Иванович совмещал работу в Институте эволюционной физиологии и биохимии РАН, где заведовал лабораторией молекулярных основ эволюции. Основная область его интересов сместилась в сторону изучения эволюции вирусов. Тогда же, в 1988 г. по предложению Министра здравоохранения СССР академика Е.И. Чазова О.И. Киселев был назначен директором НИИ гриппа. Вместе с назначением было подписано постановление о переводе всего отделения генной инженерии из Министерства медицинской и микробиологической промышленности в структуру НИИ гриппа.

В 1998 г. Олег Иванович получил звание профессора по специальности «Биохимия», в 2000 г. — избран членом-корреспондентом РАМН, а в 2005 г. — академиком РАМН по специальности «Молекулярная вирусология» (с 2013 г. — академик РАН).

За время работы в НИИ гриппа основные направления научных исследований Олега Ивановича относились к области молекулярной биологии вирусов, генной инженерии иммунобиологических препаратов, дизайну и исследованию молекулярных механизмов действия химиопрепаратов. Им создана своя научная школа по молекулярной вирусологии, под его руководством защищены 11 кандидатских и 4 докторских диссертации, опубликовано более 350 научных работ, включая 20 монографий, 45 патентов на изобретения и 11 сборников.

Олег Иванович систематизировал современные представления о потенциальных лекарственных мишенях в репликативном цикле вирусов, внёс вклад в молекулярно-генетическое обоснование терапии гриппа этиотропными препаратами. Благодаря сотрудничеству с Институтом органического синтеза УрО РАН и Уральским федеральным университетом было разработано новое поколение противовирусных препаратов на основе нитроазолоазинов, проведён скрининг соединений данного класса и осуществлён выбор соединения — лидера препарата триазавирин, проведены уникальные работы по расшифровке механизма действия препарата.

Олег Иванович внёс важнейший вклад в разработку иммунобиологических препаратов против гриппа и туберкулёза, лично выступал как популя-

ризатор широкого внедрения в практику вакцинопрофилактики гриппа и других инфекционных болезней.

Академик О.И. Киселев плодотворно сотрудничал с зарубежными научными коллективами и развивал международное сотрудничество НИИ гриппа; был одним из ведущих специалистов в области биологической безопасности и биотерроризма. Внёс значительный вклад в создание проектов по линии Российско-Вьетнамского тропического центра.

Отдельно необходимо отметить его значительный вклад в развитие сотрудничества с Всемирной организацией здравоохранения по вопросам надзора и контроля за гриппом и острыми респираторными заболеваниями на территории России. Длительная работа в составе комиссий ВОЗ по подготовке к пандемии гриппа способствовала укреплению авторитета и признанию России как страны с высоким уровнем решения проблем защиты населения от пандемии гриппа, ведущим в мире производителем противогриппозных вакцин и противовирусных препаратов.

Заслуги Олега Ивановича Киселева по достоинству были оценены государством: он лауреат премии Правительства РФ 2004 г. в области науки и техники; в 2004 г. награждён медалью Ордена «За заслуги перед Отечеством» II степени; в 2014 г. получил звание заслуженного деятеля науки Российской Федерации.

Олег Иванович активно готовил высококвалифицированные научные кадры и оставил после себя целую плеяду учеников, продолживших традиции его школы молекулярной вирусологии. Они считают делом чести сохранить и преумножить наследие академика О.И. Киселева и всё самое ценное из него передать следующим поколениям сотрудников Института и своим ученикам. Все они видят своё призвание в решении актуальных проблем здравоохранения.

Академик О.И. Киселев, безвременно ушедший 24 ноября 2015 г., — один из наиболее ярких представителей медицинской науки, объединявший в себе фундаментальные представления о медицине и биологии, внёс существенный вклад в решение многих фундаментальных и прикладных задач в области молекулярной вирусологии, генной инженерии, химиотерапии, профилактики и эпидемиологии гриппа и других вирусных инфекций. Коллеги вспоминают его с восхищением и теплотой как человека, который любил и умел жить, подавал самоотверженный пример настоящего учёного, выполнявшего свой долг, несмотря ни на какие трудности, отстаивал интересы страны на международной арене и развивал научную кооперацию с ведущими учёными по всему миру.

Светлая память академику О.И. Киселеву.

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