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* The article is published in Russian and English on the journal's website: https://www.microbiol.crie.ru.

Original Study Article
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Genetic characteristics of influenza A and B viruses circulating in Russia in 2019–2023

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Abstract

Relevance. Influenza viruses have a high potential for genetic change. These viruses are monitored annually around the world, including Russia, to determine the dominant genetic groups and select the strains to be included in influenza vaccines.

Objectives of the study include: analysis of influenza virus circulation in Russia in 2019–2023, phylogenetic and molecular analysis of hemagglutinin (HA) sequences of influenza viruses, detection of mutations associated with drug resistance to neuraminidase (NA) inhibitors and M2-protein (M2) ion channel inhibitors.

Materials and methods. Biological samples containing RNA of influenza viruses were studied: 410 A(H1N1) pdm09, 147 A(H3N2) and 167 B(Victoria). Sequencing of the HA, NA, M fragments was performed on the 3500xL Genetic Analyzer (Applied Biosytems). Data processing and analysis were carried out using DNASTAR, Nextclade, FluSurver and BioNumerics v.6.6 software.

Results. Influenza A(H1N1)pdm09, A(H3N2), B(Victoria) viruses circulating in 2019-2023 were investigated. The highest variability of HA was observed in A(H3N2) viruses. All influenza A(H1N1)pdm09 viruses in the 2022–2023 season had a previously unknown mutation *E224A* in HA, which increases its affinity for α-2,3 sialic acids — receptors localized in the human lungs, to which the virus binds via HA. 2 and 3% of influenza A(H1N1)pdm09 viruses in 2019–2020 and 2022–2023, respectively, had the *D222N* mutation in the receptor-binding site of HA, which is associated with more severe disease. The oseltamivir and zanamivir resistance mutation *H275Y* in NA was detected in 2.3% of influenza A(H1N1)pdm09 viruses in 2022–2023. No oseltamivir and zanamivir resistance mutations in NA were detected in all tested influenza A(H3N2) and B viruses. Sequencing data revealed a mutation of adamantane resistance *S31N* in M2 in all studied influenza viruses A(H1N1)pdm09 and A(H3N2).

Conclusions. The detection of amino acid substitutions in HA antigenic sites and resistance mutations in NA and M2 confirms the evolution of influenza viruses and the necessity for continuous genetic surveillance. The vast majority of currently circulating viruses remain sensitive to NA inhibitors.

Keywords: *influenza viruses, sequencing, phylogenetic analysis, molecular genetic analysis*

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 for Epidemiology (protocol No. 3, March 27, 2020).

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Оригинальное исследование
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Генетическая характеристика вирусов гриппа A и B, циркулировавших в России в 2019–2023 годах

Яцышина С.Б.✉, Артамонова А.А., Елькина М.А., Валдохина А.В., Буланенко В.П., Берсенева А.А., Акимкин В.Г.

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Аннотация

Актуальность. Вирусы гриппа обладают высоким потенциалом генетических изменений. Ежегодно по всему миру, в том числе в России, проводится мониторинг вирусов гриппа, чтобы определить доминирующие генетические группы и отобрать среди них штаммы, которые войдут в состав противогриппозных вакцин.

Цели исследования: анализ циркуляции вирусов гриппа в России в 2019–2023 гг., проведение филогенетического и молекулярного анализа последовательностей гемагглютинина (HA) вирусов гриппа, выявление мутаций резистентности к ингибиторам нейраминидазы (NA) и ингибиторам ионного канала M2-белка (M2).

Материалы и методы. Исследованы биологические образцы, содержащие РНК вирусов гриппа: 410 A(H1N1)pdm09, 147 A(H3N2) и 167 B(Виктория). Осуществляли секвенирование фрагментов сегментов HA, NA, M, проводили обработку и анализ данных.

Результаты. Исследованы нуклеотидные последовательности HA, NA, M вирусов гриппа A(H1N1)pdm09, A(H3N2) и B(Виктория), циркулировавших в 2019–2023 гг. Наибольшая вариабельность HA наблюдалась у вирусов A(H3N2). Все вирусы гриппа A(H1N1)pdm09 сезона 2022–2023 гг. имели не встречавшуюся ранее мутацию *E224A* в HA, которая увеличивает его сродство к α-2,3-сиаловым кислотам — рецепторам, локализованным в лёгких человека, с которыми связывается вирус посредством HA. У 2 и 3% вирусов A(H1N1)pdm09 в сезонах 2019–2020 и 2022–2023 гг. соответственно в рецептор-связывающем сайте HA обнаружена мутация *D222N*, которая ассоциирована с более тяжёлым заболеванием. Мутация устойчивости к осельтамивиру и занамивиру *H275Y* в NA выявлена у 2,3% вирусов гриппа A(H1N1)pdm09 в 2022–2023 гг. Во всех исследованных вирусах гриппа A(H3N2) и B мутации устойчивости к осельтамивиру и занамивиру в NA не обнаружены. По данным секвенирования, во всех исследованных вирусах гриппа A(H1N1)pdm09 и A(H3N2) имелась мутация устойчивости к адамантанам *S31N* в M2.

Выводы. Обнаружение мутаций, затрагивающих антигенные и рецептор-связывающие сайты HA, а также мутаций резистентности в NA и M2 подтверждает необходимость постоянного генетического надзора за вирусами гриппа. Подавляющее большинство циркулирующих в настоящее время вирусов сохраняют чувствительность к ингибиторам NA.

Ключевые слова: *вирусы гриппа, секвенирование, филогенетический анализ, молекулярно-генетический анализ*

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Introduction

Influenza viruses are enveloped viruses of the *Orthomyxoviridae* family, which are classified into 4 genera: influenza A, B, C and D viruses. Influenza A and B viruses pose the most significant danger to human health [1].

Influenza A viruses are the cause of most annual epidemics and all recurrent human pandemic diseases. They are subdivided into subtypes according to the combination of 2 surface glycoproteins located in the lipid membrane of virions: hemagglutinin (HA) and neuraminidase (NA). In birds, the main natural reservoir of influenza A viruses, 16 HA and 9 NA have been described; another 2 HA and NA have been found in bats [2]. Currently, influenza A viruses of the H1N1pdm09 and H3N2 subtypes cause the greatest number of epidemic diseases in humans[1].

Influenza B viruses are specific to humans (cases in seals have also been described [3]) and are divided into 2 evolutionary lineages with significantly different antigenic properties (B/Victoria and B/Yamagata).

Influenza viruses are in a state of continuous evolution in their reservoirs, facilitated by a high mutation rate due to the absence of the proofreading activity of RNA-dependent RNA polymerase [4]. The main reservoir for influenza A viruses is wild migratory birds and other mammals, including humans, while influenza B and C viruses have no reservoir in the wild. Cumulative changes in sequences encoding HA and NA lead to antigenic drift of influenza A and B viruses: the structure of antigenic surfaces recognized by specific antibodies changes, contributing to the annual epidemic [5]. Antigenic shift is also possible for influenza A viruses: segments encoding HA (and to a lesser extent NA) that have evolved in animal influenza viruses can combine with human influenza virus segments to form new reassortant strains capable of causing pandemics [4, 5]. Since 1889, there have been 5 known pandemics of influenza A viruses, the most serious of which in 1918 was caused by the H1N1 subtype and the most recent in 2009 by the A(H1N1)pdm09 subtype [4, 6]. The H3N2 subtype began circulating in the 1968 pandemic [7]. Since 1971, both subtypes of influenza A virus — H3N2 and H1N1 (since 2009 — H1N1pdm09) and both antigenic lineages of influenza B virus have circulated annually with greater or lesser intensity.

Influenza B virus was identified in 1940.Two antigenic virus lineages, B/Victoria/2/87-like (Victoria) and B/Yamagata/16/88-like (Yamagata), have been co-circulating since 1983 [8].

B/Victoria influenza viruses evolve more rapidly with greater positive selection pressure than B/Yamagata lineage viruses [9], and the latter have not circulated since March 2020: since then, there have been sporadic reports of B/Yamagata influenza viruses, but without confirmation by HA sequencing of the virus[2].

Under the auspices of WHO, influenza viruses are monitored worldwide, including their typing, genetic characterization and antigenic properties, to select among the predominant antigenic groups of virus strains to be included in influenza vaccines in the next epidemic season. The selection of vaccine strains with specific immunogenic properties is necessary to ensure an immune response against influenza viruses [10]. Large-scale genetic monitoring allows deep differentiation into clades and subclades and defines the trend of influenza virus evolution: the emergence, spread and disappearance from circulation of certain genetic variants.

During evolution, the most significant changes affect the HA of influenza viruses. The HA glycoprotein of influenza viruses is synthesized as a single polypeptide chain, which is further proteolytically cleaved into 2 subunits: HA1 and HA2. HA1 is responsible for binding the virus to sialic acids (SAs) on the cell membrane surface, while HA2 ensures fusion of the virus and endosome membranes.

Antigenic sites located at the HA1 apex near the receptor-binding site are the main targets for human neutralizing antibodies. HA glycosylation is associated with many properties, including immunogenicity and receptor specificity, and plays an important role in protecting antigenic sites from neutralizing antibodies [11]. The glycosylation pattern is more variable in HA1 than in HA2, which is more conserved [12].

Amino acid substitutions in the receptor-binding domain of HA affect the ability to bind to the host cell surface, which alters the virulence of the virus [13]. On the surface of human upper respiratory tract cells, predominantly SA-α2,6 are located, whereas in the lower respiratory tract, SA-α2,3 are located. Increased affinity of the virus for α-2,3-SAs may increase the severity of the disease [14].

The evolution of influenza B/Victoria viruses, in addition to point mutations, is facilitated by insertions and deletions of amino acids in the HA receptor binding region, which leads to immune evasion [15].

Amino acid substitutions in NA (influenza A and B viruses) and M2 (influenza A viruses) reduce the efficacy of drugs: NA inhibitors (oseltamivir, zanamivir) and adamantanes (amantadine and rimantadine), inhibitors of the M2 ion channel. According to the U.S. Centers for Disease Control and Prevention, virtually 100% of influenza A viruses are currently resistant to amantadine and rimantadine [16].

[1] WHO. Influenza (seasonal). URL: https://www.who.int/ru/news-room/fact-sheets/detail/influenza-(seasonal)

[2] ECDC. Influenza virus characterisation, Summary Europe, July 2021. Stockholm; 2021. URL: https://www.ecdc.europa.eu/en/publications-data/influenza-virus-characterisation-summary-europe-july-2021; ECDC. Influenza virus characterization: summary report, Europe, July 2022. Copenhagen; 2022. URL: https://www.ecdc.europa.eu/en/publications-data/influenza-virus-characterization-summary-europe-july-2022

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The objectives of this study are the molecular genetic analysis of HA, NA and M segments of influenza A(H1N1)pdm09, A(H3N2) and B/Victoria viruses circulating in Russia during the epidemic seasons 2019–2023, identification of amino acid substitutions in HA compared to vaccine strains, their possible impact on antigenic properties and strength of binding to specific receptors; phylogenetic analysis on the HA gene sequences, as well as analysis of NA and M2 for the presence of molecular markers of resistance to antiviral drugs.

Materials and methods

Detection of influenza A and B virus RNA in biological material (nasopharyngeal and oropharyngeal swabs, sputum, tracheal aspirates, bronchoalveolar lavage) obtained as a result of routine monitoring for influenza viruses was carried out in laboratories of the Hygiene and Epidemiology Centers of Rospotrebnadzor in 50 regions of the Russian Federation (Central, Northwestern, Southern, North Caucasus, Volga, Urals, and Far Eastern federal districts) by polymerase chain reaction (PCR) with hybridization-fluorescence detection of amplification products. 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 for Epidemiology (protocol No. 3, March 27, 2020). In case of unfavorable outcome of the disease, postmortem material (lung autopsy specimens) was examined.

For molecular genetic analysis by nucleic acid sequencing, biological material was submitted to the Reference Center for monitoring of upper and lower respiratory tract infections at the Laboratory of Molecular Diagnostics and Epidemiology of Respiratory Tract Infections of the Central Research Institute of Epidemiology.

Extraction of influenza virus RNA from biological material and the subsequent reverse transcription reaction were performed using the RIBO-prep and REVERTA-L reagent kits (AmpliSens). PCR for influenza virus RNA detection was performed using AmpliSens Influenza virus A/B, AmpliSens Influenza virus A-type-FL, AmpliSens Influenza virus A/H1-swine-FL and AmpliSens Influenza virus B-type-FL reagent kits (produced by the Central Research Institute of Epidemiology).

To amplify fragments of HA, NA, M genes of influenza viruses A/H1N1pdm09 and A/H3N2, HA and NA genes of influenza B viruses, PCR with electrophoresis detection was performed on thermocyclers (DNA-Technology) using AmpliSens reagents (Central Research Institute of Epidemiology).

Amplified fragments of individual segments of influenza viruses (NA, NA, M) were sequenced in the Scientific Group of Genetic Engineering and Biotechnology of the Central Research Institute of Epidemiology by the Sanger method using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems by Thermo Fisher Scientific) on the 3500xL Genetic Analyzer sequencer (Applied Biosystems).

Sequencing results were analyzed in the Reference Center for monitoring of upper and lower respiratory tract infections using the DNASTAR software block (SeqMan, EditSeq, MegAlign). The obtained nucleotide sequences were uploaded to the international database GISAID, can be filtered by CRIE Search patterns. Phylogenetic analysis was performed using the BioNumerics v. 6.6 program with the UPGMA method. Nextclade, FluSurver online platforms were used to track amino acid mutations. Amino acid numbering is given according to the corresponding influenza virus subtype.

Results

Biological samples containing RNA of influenza viruses: 410 A(H1N1)pdm09, 147 A(H3N2), and 167 B(Victoria) circulating in the epidemic seasons 2019–2020, 2020–2021, 2021–2022, 2022–2023 were analyzed. Influenza A(H1N1)pdm09 and influenza B virus were both detected in 1 sample (upper respiratory swabs) from the 2022–2023 season. **Figure 1** shows that the number of influenza viruses tested varied in different years, which is due to the different intensity of the influenza epidemic process in different seasons.

The nucleotide sequences of NA, HA, M genes of influenza A(H1N1)pdm09 and A(H3N2) viruses, and NA and HA genes of influenza B virus were obtained and analyzed (**Table 1**).

Phylogenetic and molecular genetic analysis of influenza viruses were performed. Clustering of influenza viruses into clades and subclades was carried out on the basis of HA nucleotide sequences; for comparison, the sequences of vaccine strains, recommended in each season for the Northern Hemisphere, cultured in chicken embryos were used, since such vaccines are widely used in Russia.

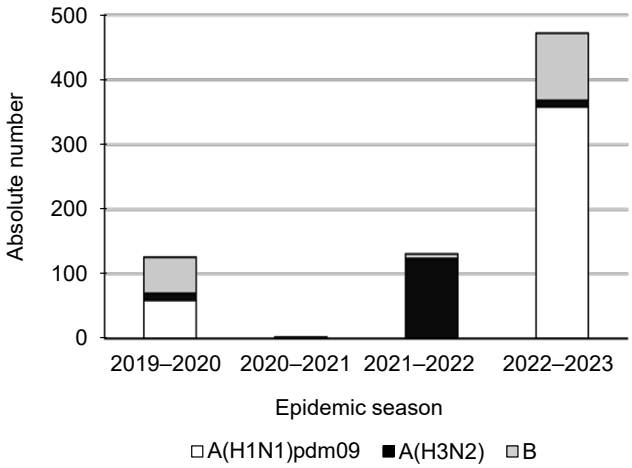

Fig. 1. Number of influenza viruses tested at the Reference center for monitoring upper and lower respiratory tract infections in 2019–2023.

Table 1. Number of sequenced segments

Influenza virus	Segment	Number (from the recovered/from the deceased)			
		2019–2020	2020–2021	2021–2022	2022–2023
(H1N1)pdm09	HA	59 (55/4)	–	–	351 (296/55)
	NA	59 (55/4)	–	–	87 (69/18)
	M	59 (55/4)	–	–	28 (16/2)
A(H3N2)	HA	11 (11/0)	2 (1/0)	124 (124/0)	10 (10/0)
	NA	2 (2/0)	1 (1/0)	44 (44/0)	1 (1/0)
	M	1 (1/0)	2 (1/0)	34 (34/0)	1 (1/0)
B	HA	56 (53/3)	–	7 (7/0)	104 (102/2)
	NA	35 (34/1)	–	1 (1/0)	36 (36/0)

Influenza A(H1N1)pdm09 viruses

The distribution of sequenced viruses into genetic clusters (clades, subclades and subgroups) and the results of comparison with the vaccine strain of each season are presented in **Table 2**. The homology of the nucleotide sequences of the HA gene of the studied viruses and vaccine strains varied in the range of 99.2–97.4% depending on the genetic cluster affiliation. The largest differences (up to 2.6%) were observed in the 2019–2020 season.

Based on sequencing results, A(H1N1)pdm09 viruses from the 2019-2020 season belonged to subclade 6B.1A.5 subgroups 5a, 5a.1, 5a.2, and subclade 6B.1A.7. This season, viruses from subclade 6B.1A.5 subgroup 5a (66%), characterized by amino acid substitutions *N129D* and *T185A* in HA1, prevailed. Subgroup 6B.1A.5a.1, characterized by amino acid substitutions *D187A*, *Q189E*, included 31% of the studied viruses. One virus belonged to subgroup 6B.1A.5a.2 (amino acid substitutions *N156K*, *L161I*, *V250A*), 1 virus belonged to subclade 6B.1A.7 (amino acid substitutions *K302T* in HA1 and *I77M*, *N169S*, *E179D* in HA2).

The A/Brisbane/02/2018 strain recommended for inclusion in the vaccine for the 2019–2020 epidemic season for the Northern Hemisphere belonged to subclade 6B.1A.1.

In the 2020–2021 and 2021–2022 seasons, influenza A(H1N1)pdm09 viruses were not sent to the reference center because they were not circulating.

All tested A(H1N1)pdm09 viruses from the 2022–2023 season belonged to clade 6B.1A.5a.2a, characterized by amino acid substitutions *K54Q*, *A186T*, *Q189E*, *E224A*, *R259K*, and *K308R*. This season's vaccine strain A/Victoria/2570/2019 was assigned to subgroup 6B.1A.5a.2.

The results of phylogenetic analysis on the HA gene of influenza A subtype H1N1pdm09 viruses in the 2019–2023 seasons are shown in **Figure 2**. The dendrogram contains a set of selected sequences of HA, which represented genetic diversity of circulated influenza viruses.

Five antigenic sites are identified in the HA molecule of influenza A(H1N1)pdm09 virus, which include the following amino acid positions: Sa (121–122 and 150–162), Sb (184–195), Ca1 (163–167, 200–202 and 232–235), Ca2 (133–139 and 218–219), Cb (67–72) [17].

From 7 to 12 mutations were detected in the HA amino acid sequence of 2019-2020 viruses compared to the vaccine strain A/Brisbane/02/2018. Of these, 7 were located in antigenic sites: Sa — *S121I/N* ($n = 2$), *N156K* ($n = 1$), *L161I* ($n = 1$), Sb — *T185I* ($n = 57$), *D187A* ($n = 18$), *Q189E* ($n = 16$), *S190N* ($n = 1$). An *R205K* substitution was found near the Ca1 antigenic site ($n = 2$). The mutations *D187A* ($n = 18$), *R221K* ($n = 2$), and *D222N* ($n = 1$) were located in the receptor-binding site. One sample belonged to subclade 6B.1A7 and carried amino acid substitutions *E68D* (in Cb), *T120A*, *S121N* (in Sa1), *R223Q*, *K302T* (in HA1), and *I77M*, *N169S*, *E179D* (in HA2).

Table 2. Results of nucleotide sequence analysis of HA influenza A(H1N1)pdm09 viruses received by the Reference center in 2019–2023

Epidemic season, years	Vaccine strain (genetic cluster)	Number of samples studied	Genetic cluster	*HA* gene homology with the vaccine strain, %
2019–2020	A/Brisbane/02/2018 (6B.1A.1)	39	6B.1A.5a	98.4–99.2
		18	6B.1A.5a.1	98.2–98.6
		1	6B.1A.5a.2	99.2
		1	6B.1A.7	98.2
2022–2023	A/Victoria/2570/2019 (6B.1A.5a.2)	351	6B.1A.5a.2a	97.4–98.9

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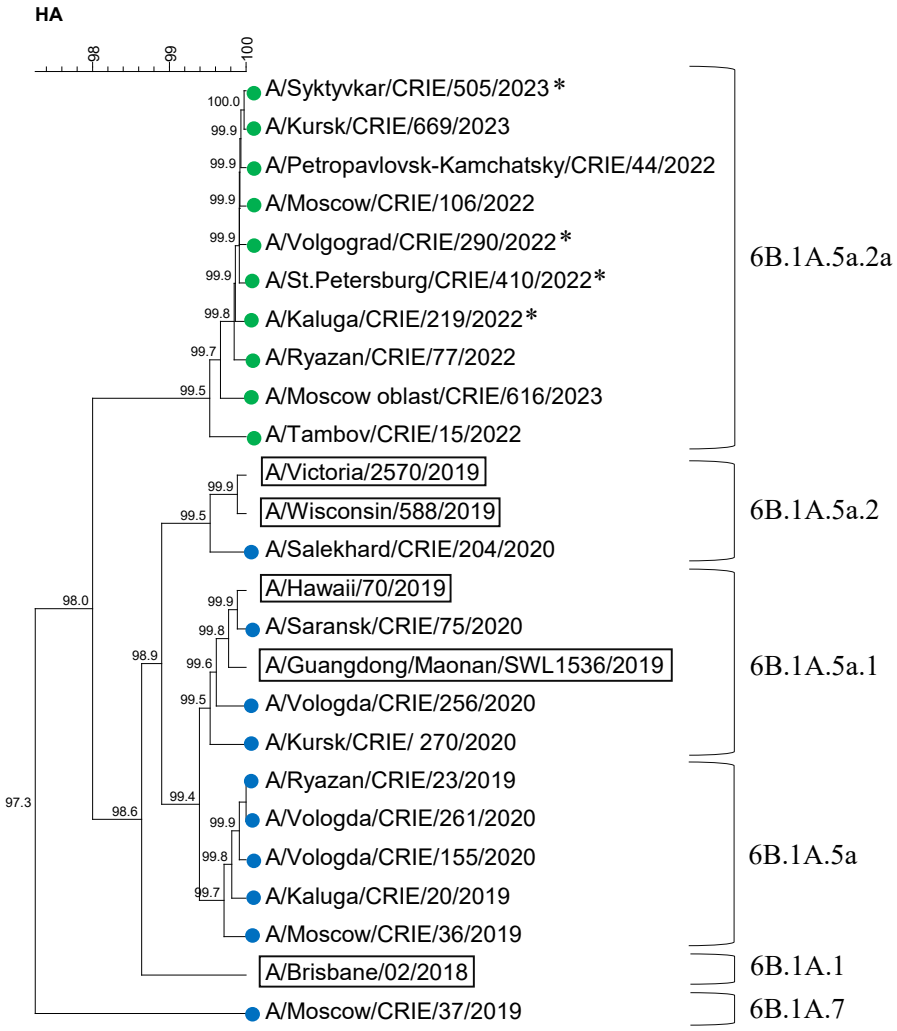

Fig. 2. Dendrogram of the HA gene of influenza A(H1N1)pdm09 viruses (data of the CRIE Reference center, 2019–2020, 2022–2023).

Vaccine strains are indicated by rectangles. Blue and green circles indicate the sequences of viruses circulating in 2019–2020 and 2022–2023 influenza seasons, respectively.
Fatal cases are marked with an asterisk.

Influenza A(H1N1)pdm09 2022–2023 viruses relative to the vaccine strain contained 6-12 amino acid substitutions. *R259K*, *K54Q*, *A186T*, *Q189E*, *E224A*, *K308R/G* substitutions in HA1 were detected more frequently. Some of the mutations affected antigenic sites: Sa — *S121N* (n = 1), *S122L* (n = 1), *K154R* (n = 1), *G155E* (n = 1), *N162S* (n = 1, loss of glycosylation site), Sb — *A186T* (n = 351), *D187V* (n = 1), *Q189E* (n = 351), *N194S* (n = 1), Ca1 – *V234I* (n = 1), Cb — *S69P* (n = 1), *L70F* (n = 1). Amino acid substitutions *N125H* (n = 1), *R205K* (n = 3) were found adjacent to antigenic sites. The *D187V* (n = 1) and *D222N* (n = 12) mutations were located in the receptor-binding site.The *D145N* mutation in HA2 resulting in an additional glycosylation site was found in 2 virus samples.

In 2019–2020, all influenza A(H1N1)pdm09 viruses lacked mutations conferring resistance to oseltamivir and zanamivir, whereas in 2022–2023 viruses with the H275Y mutation in the NA accounted for 2.3% and were detected in respiratory swabs of unvaccinated patients from the Arkhangelsk region in December 2022. All A(H1N1)pdm09 influenza viruses for which the M gene sequence was obtained were resistant to adamantanes (*S31N* mutation in M2).

Influenza A(H3N2) viruses

The distribution of the studied influenza A(H3N2) viruses by genetic clusters and the results of comparison with the vaccine strain according to each season are presented in **Table 3**. The homology of the nucleotide sequences of the *HA* gene of the studied viruses and vaccine strains varied in the range of 99.0–95.8% depending on the genetic cluster affiliation. Maximum differences (3.6–4.2%) were observed in the 2019–2020 season.

Influenza A(H3N2) viruses from the 2019–2020 season were categorized as group 3C.2a1b, clusters 3C.2a1b.1b and 3C.2a1b.2a. Vaccine strain A/Kansas/14/2017 was assigned to cluster 3C.3a.1 (characterized by amino acid substitutions at positions *S91N*, *N144K*, the latter resulting in the loss of a potential glycosylation site, *F193S* in HA1 and *D160N* in HA2). Cluster 3C.2a1b.1b, characterized by amino acid substitutions at positions *S137F*, *A138S*, and *F193S* in HA1, included 64% of the samples. The cluster 3C.2a1b.2a, characterized by amino acid substitutions at positions *K83E*, *Y94N* in HA1 and *I193M* in HA2, included 36% of the samples.

In the hemagglutinin molecule of influenza A(H3N2) virus, the following amino acid positions are considered to be antigenic sites:

- A (122, 124, 126, 130–133, 135, 137, 138, 140, 142–146, 150, 152, 168);
- B (128, 129, 155–160, 163–165, 186–190, 192–194, 196–198);
- C (44–48, 50, 51, 53, 54, 273, 275, 276, 278–280, 294, 297, 299, 300, 304, 305, 307–312);
- D (96, 102, 103, 117, 121, 167, 170–177, 179, 182, 201, 203, 207–209, 212–219, 226–230, 238, 240, 242, 244, 246–248);
- E (57, 59, 62, 63, 67, 75, 78, 80–83, 86–88, 91, 92, 94, 109, 260–262, 265) [18].

Three antigenic sites overlap with the receptor-binding site: site A with loop 130 (135, 136, 137, 138, 153); site B with helix 190 (186, 190, 194, 195); and site D with loop 220 (226 and 228) [19].

Both influenza A(H3N2) viruses of the 2020–2021 season belonged to subclade 2a.2 of subgroup 3C.2a1b.2a.2a.2a.2, which is characterized by amino acid substitutions *Y159N*, *T160I* (loss of glycosylation site, *L164Q*, *G186D*, *D190N*) in HA1, whereas vaccine strain A/Hong Kong/2671/2019 belonged to cluster 3C.2a1b.1b.

In the 2021–2022 season, as in the previous season, all influenza A(H3N2) viruses belonged to subcluster 3C.2a1b.2a.2. In the 2022–2023 classification, viruses were assigned to 4 subclades: viruses of 3C.2a1b.2a.2a.2 (amino acid substitutions *D53G*, *R201L*, *S219Y*) prevailed, 3C.2a1b.2a.2c (amino acid substitutions *S205F*, *A212T*) were less frequently found, and 3C.2a1b.2a.2a.2 and 3C.2a1b.2a.2a.2a.1 (amino acid substitutions *D53G*, *D104G*, *K276R*) were even less frequently found. The vaccine strain A/Cambodia/e0826360/2020 belonged to group 3C.2a1b.2a, but to a different genetic subgroup 3C.2a1b.2a.1a, characterized by amino acid substitutions *L157I*, *K220R*.

Influenza A(H3N2) viruses from the 2022-2023 season belonged to clade 3C.2a1b.2a.2a.2, as did the vaccine strain A/Darwin/9/2021, which was in the vaccine administered in the Northern Hemisphere in 2022–2023. Within the clade, the viruses were differentiated into subclades: 3 belonged to 3C.2a1b.2a.2a.3a.1 (amino acid substitution *I140K*), 3 belonged to 3C.2a1b.2a.2b (characterized by amino acid substitutions *E50K*, *F79V*, *I140K*), and 2 belonged to 3C.2a1b.2a.2a.2a.1b (amino acid substitutions *I140K*, *R299K*). Only 2 HAs were close to the vaccine strain A/Darwin/9/2021 (subclade 3C.2a1b.2a.2a, characterized by amino acid substitution *H156S*).

The results of phylogenetic analysis on the HA gene of influenza A subtype H3N2 viruses are presented in **Fig. 3**. In the 2019–2020 season, 21–23 mutations were detected in the HA sequence of viruses compared to the vaccine strain A/Kansas/14/2017, 19 of these mutations located in antigenic sites:

- A — *T131K* (*n* = 3), *T135K* (*n* = 7), *S137F* (*n* = 7), *I140K* (*n* = 7), *K144S* (*n* = 10);
- B — *A128T* (*n* = 3), *S159Y* (*n* = 10), *K160T* (*n* = 10), *N190D* (*n* = 10), *S193F* (*n* = 3);
- D — *N121K* (*n* = 10), *N171K* (*n* = 10), *V230I* (*n* = 1), *T246N* (*n* = 10);
- E — *E62G* (*n* = 10), *K83E* (*n* = 3), *N91S* (*n* = 10), *K92R* (*n* = 10), *Y94N/S* (*n* = 3).

Three substitutions were localized in the receptor-binding site: in loop 130, *T135K*, *S137F*, and in helix 190, *N190D*. The *I77V*, *M149I*, and *G155E* mutations in HA2 of 11 viruses were found.

Mutations that lead to the appearance of new potential N-glycosylation sites were found: *A128T* (in 3 viruses of subgroup 3C.2a1b.2a), *K160T* (in all 10 viruses), *T246N* (in all 10 viruses). Seven viruses of subgroup 3C.2a1b.1b had a *T135K* mutation that results in loss of the N-glycosylation site.

A(H3N2) viruses examined in the 2020–2021 season, there were 22–24 substitutions in the HA se-

Table 3. Results of HA nucleotide sequence analysis of influenza A(H3N2) viruses received by the Reference center in 2019–2023

Epidemic season. years	Vaccine strain (genetic cluster)	Number of samples studied	Genetic cluster	HA gene homology with the vaccine strain. %
2019–2020	A/Kansas/14/2017 (3C.3a.1)	7	3C.2a1b.1b	96.2–96.4
		4	3C.2a1b.2a	95.8–96.0
2020–2021	A/Hong Kong/2671/2019 (3C.2a1b.1b. previously 3C.2a1b + T135K-B)	2	3C.2a1b.2a.2a.2	97.3–97.4
2021–2022	A/Cambodia/e0826360/2020 (3C.2a1b.2a.1a)	3	3C.2a1b.2a.2	98.6–98.7
		3	3C.2a1b.2a.2a.1	98.6–98.7
		105	3C.2a1b.2a.2a.2	98.1–98.9
		13	3C.2a1b.2a.2c	98.6–98.9
2022–2023	A/Darwin/9/2021 (2a. previously 3C.2a1b.2a.2a)	2	3C.2a1b.2a.2a	99.4
		2	3C.2a1b.2a.2a.1b	98.9–99.0
		3	3C.2a1b.2a.2a.3a.1	98.5–98.6
		3	3C.2a1b.2a.2b	98.5

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quences compared to the vaccine strain A/Hong Kong/2671/2019, 16 of which were in the antigenic sites of both viruses: A — *T131K*, *K135T*, *F137S*, *S138A*; B — *A128T*, *H156S*, *Y159N*, *L164Q*, *V186D*, *D190N*; C — *D53G*; D — *R201K*, *S219Y*; E — *K83E*, *Y94N*. One virus had an *I214V* mutation in site D; 5 substitutions were localized in the receptor-binding site: in loop 130, *K135T*, *F137S*, *S138A*; and in helix 190, *V186D*, *D190N*. Additionally, *N225D*, *A128T*, and *K135T* mutations were found outside the antigenic sites in both viruses, the latter resulting in new potential N-glycosylation sites.

In the 2021–2022 season, HA amino acid sequences contained 9–12 mutations compared to the vaccine strain A/Cambodia/e0826360/2020.

The following substitutions in antigenic sites were detected in 124 viruses of all subclades: B, Y159N (1 had *Y159S*), *K160I*, *L164Q*, *R186D*, *D190N*, *P198S*; D, *N171K*. The *R186D*, *D190N* substitutions were in the receptor-binding site. Three viruses of subclade 3C.2a1b.2a.2a.2 had mutations: A — *I140K*, D — *R201I*. One virus had the *S219Y* mutation in the antigenic site D.

Three viruses of subclade 3C.2a1b.2a.2a.1 contained mutations: C — *D53G*, *K276R*; B — *H156S*; outside antigenic sites — *D104G*; 105 viruses of subclade 3C.2a1b.2a.2a.2a.2 had mutations in antigenic site D — *R201K*, *S219Y*. Total 104 viruses had an *I25V* substitution outside the antigenic sites. Four viruses had mutations that lead to loss of the glycosylation site: 3 viruses had *N122D*, 1 had *N165K*. Thirteen viruses of

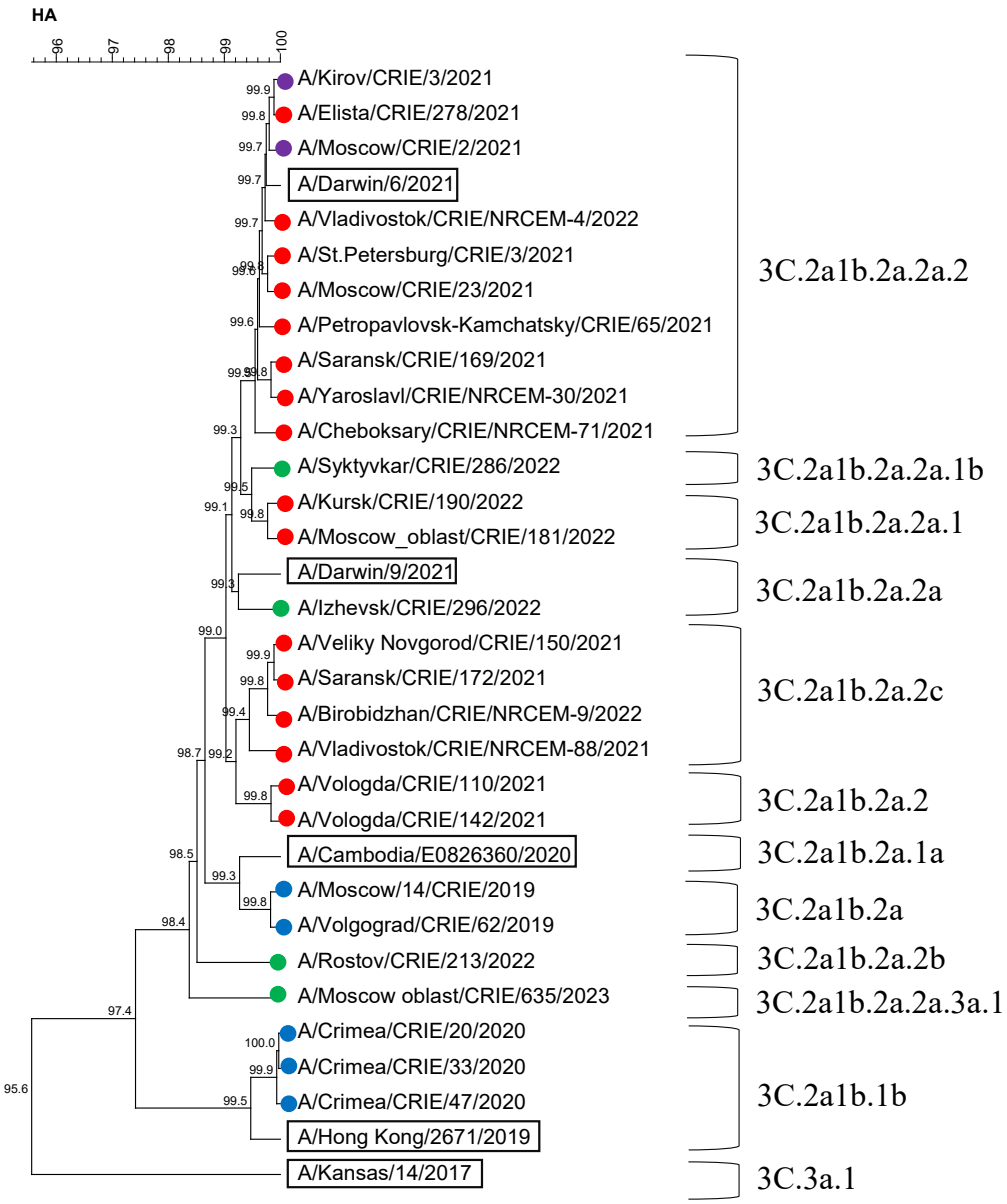

Fig. 3. Dendrogram of the HA gene of influenza A(H3N2) viruses (data from the CRIE Reference center, 2019–2023).
Vaccine strains are indicated by rectangles. Blue, violet, red and green circles indicate the sequences of viruses circulating in 2019–2020, 2020–2021, 2021–2022 and 2022–2023 influenza seasons, respectively. Fatal cases are marked with an asterisk.

subclade 3C.2a1b.2a.2c had an *A212T* mutation in antigenic site D. Twelve viruses had an *S205F* substitution outside the antigenic sites. Two viruses had an *S124N* mutation resulting in loss of the glycosylation site.

In the 2022–2023 season, the A(H3N2) viruses studied had 2–9 amino acid substitutions in HA compared with vaccine strain A/Darwin/9/2021 and 3–9 substitutions compared with A/Darwin/6/2021 (the sequence of strain A/Darwin/9/2021 differs from A/Darwin/6/2021 by the *G53D* mutation). Two viruses of subclade 3C.2a1b.2a.2a had a mutation in the D site, *I217V*. Two viruses of subclade 3C.2a1b.2a.2a.1b had mutations localized in site A — *I140K*, C — *K276R* and *R299K*. Three viruses of subclade 3C.2a1b.2a.2a.3a.1 had mutations in the C site — *E50K*, *D53N*, A — *I140K*, B — *I192F*; 3 viruses of subclade 3C.2a1b.2a.2b had *E50K* mutations in the C site and *I140K* mutations in site A. Two viruses had an amino acid substitution of *N96S* in HA1 leading to the appearence of N-glycosylation site, and one had an *N122D* mutation in HA1 leading to loss of the N-glycosylation site.

No resistance mutations to oseltamivir and zanamivir were detected in the NA of influenza A(H3N2) viruses tested for the 2019–2023 seasons, while all had the *S31N* adamantane resistance mutation in M2.

Influenza B viruses

All influenza B viruses received for study in 2019–2023 belonged to the B/Victoria lineage (according to PCR and sequencing results).

The distribution of investigated influenza B viruses by genetic clusters and the results of comparison with the vaccine strain of each season are presented in **Table 4**. The homology of the nucleotide sequences of the HA gene of the studied viruses and vaccine strains varied in the range of 99.7–98.2% depending on the genetic cluster affiliation. Maximum similarity (99.0–99.7%) was observed in the season 2022–2023.

In the 2019–2020 season, 98% of influenza B viruses belonged to the Victoria lineage subclade V1A.3 (1A(Δ3)B), characterized by triple deletion of amino acid residues 162–164 and amino acid substitutions *K136E*, *G133R* in HA; the remainder belonged to the subgroup V1A.3a.1, characterized by amino acid substitutions in HA1 *V117I*, *V220M*. The vaccine strain B/Colorado/06/2017 for the 2019–2020 Northern Hemisphere epidemic season belonged to subclade V1A.1 (1A(D 2)B) of the Victoria lineage, characterized by double deletion of amino acid residues 162–163 and amino acid substitutions at positions *D129G*, *I180V* in HA1, *R151K* in HA2.

In the 2021–2022 season, 7 influenza B viruses belonged to the Victoria lineage subclade V1A.3, subgroup V1A.3a.2 (characterized by amino acid substitutions at HA1 positions *A127T*, *P144L*, *K203R*). Vaccine strain B/Washington/02/2019, which was included in the 2021–2022 Northern Hemisphere vaccines, belonged to the Victoria lineage of subclade V1A.3.

In the 2022–2023 season, all influenza B viruses belonged to lineage Victoria subclade V.1A.3a.2. Vaccine strain B/Austria/1359417/2021, which was included in vaccines in Russia in 2022–2023, also belonged to lineage Victoria subclade V.1A.3a.2. The results of phylogenetic analysis on the HA gene of influenza B viruses of lineage Victoria are presented in **Fig. 4**.

There are 4 antigenic sites in the HA molecule of influenza B virus: loop 120 and adjacent regions (116–137), loop 150 (141–150), loop 160 (162–167), helix 190 and its surrounding regions (194–202) [20]. The receptor-binding site is formed by helix 190 (193–202), loop 240 (237–242), loop 140 (136–143) [21].

In the 2019–2020 season, the HA amino acid sequences of viruses had 8–12 mutations compared to the vaccine strain B/Colorado/06/2017, as well as an additional amino acid deletion at position 164. There were 11 substitutions in antigenic sites: *I117V* (*n* = 2), *R118K* (*n* = 2), *N126K* (*n* = 5), *A127T* (*n* = 1), *E128K* (*n* = 1), *D129N* (*n* = 55), *G133R* (*n* = 50), *Y135D* (*n* = 1), *K136E* (*n* = 56) in loop 120; *N166D* (*n* = 1) in loop 160; N197D (*n* = 1) in helix 190. Three substitutions were in the receptor-binding site: in helix 190, *N197D*; in loop 240, *P241Q* (*n* = 1); in loop 140, *K136E*. One virus each had *N166D*, *N197D*, and *N233S* substitutions that result in loss of the glycosylation site.

Influenza B viruses in the 2021–2022 season had 8–9 amino acid substitutions in HA compared to the vaccine strain B/Washington/02/2019. Amino acid substitutions were located in 5 positions of antigenic sites: in loop 120, *A127T* (*n* = 7), *R133G* (*n* = 7); in

Table 4. Results of HA nucleotide sequence analysis of influenza B viruses of the Victoria lineage received by the Reference center in 2019–2023

Epidemic season	Vaccine strain (genetic cluster)	Number of samples studied	Genetic cluster	*HA* gene homology with the vaccine strain. %
2019–2020	B/Colorado/06/2017 (V1A.1. previously 1A(Δ2)B)	55	V1A.3	98.2–98.8
		1	V1A.3a.1	98.6
2021–2022	B/Washington/02/2019 (V1A.3. previously 1A(Δ3)B)	7	V1A.3a.2	98.2–98.7
2022–2023	B/Austria/1359417/2021 (V1A.3a.2)	104	V1A.3a.2	99.0–99.7

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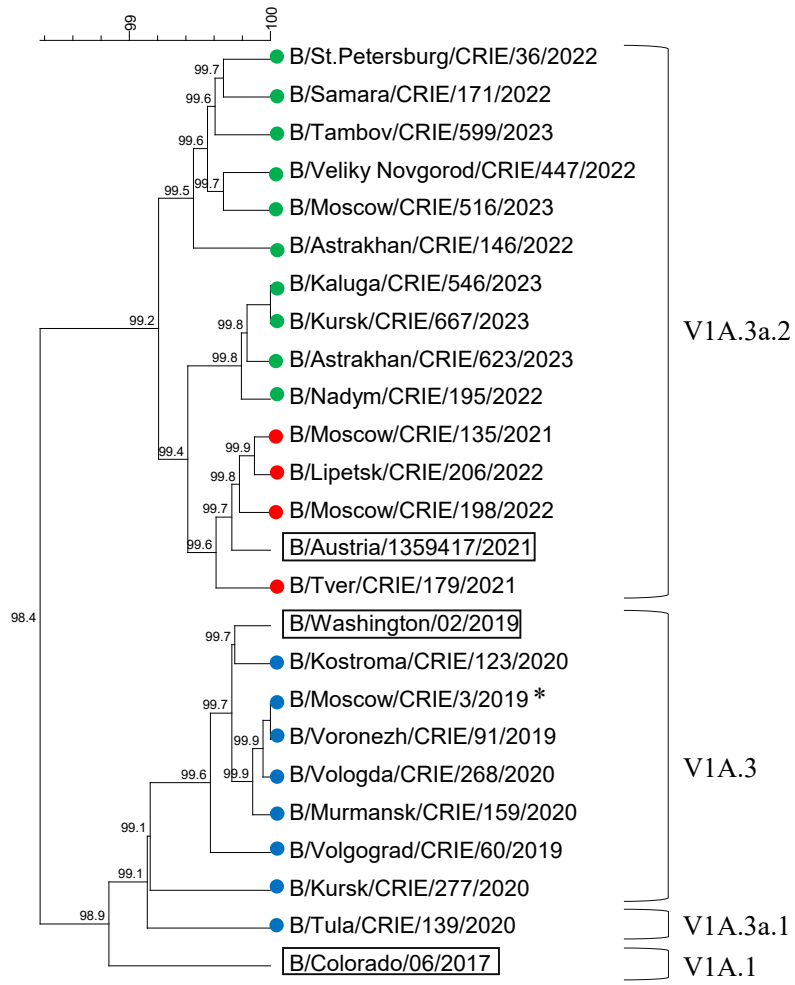

Fig. 4. Dendrogram of the HA gene of influenza B viruses of the Victoria lineage.

Data from the CRIE Reference center, 2019–2020, 2020–2021, 2022–2023. Vaccine strains are indicated by rectangles. Blue, red and green circles indicate the sequences of viruses circulating in 2019–2020, 2021–2022 and 2022–2023 influenza seasons, respectively.
Fatal cases are marked with an asterisk.

loop 150, *P144L* (n = 7), *N150K* (n = 7); and in helix 190, *N197D/E* (n = 7). One substitution was in the receptor-binding site, in helix 190 – *N197D/E*. Mutations *N197D* observed in 6 viruses and *N197E* identified in 1 virus result in loss of the glycosylation site.

There were 1–6 mutations in the HA of influenza B viruses in the 2022–2023 season compared to the vaccine strain B/Austria/1359417/2021. Seven substitutions were in antigenic sites: in loop 120 — *T121N* (n = 25), *H122N* (n = 3), *E128K* (n = 67), in loop 150 — *G149E* (n = 1), in helix 190 — *D197E* (n = 40), *T199A/I* (n = 100). Two substitutions were in the receptor-binding site, in helix 190 — *D197E*, *T199A/I*. In one virus, a *T196I* substitution was found in HA2 that resulted in loss of the glycosylation site.

No mutations in the NA gene that reduce sensitivity to oseltamivir and zanamivir were found in the examined influenza B viruses of the 2019–2023 seasons.

Discussion

The etiologic structure of influenza varied in different epidemic seasons in Russia and globally. The samples containing influenza viruses submitted to the Reference Center for study were randomly selected in Russian regions during patient screening, so we can assume that their spectrum and genetic characteristics reflect general patterns across the country and allow us to judge the structure of influenza and genetic diversity of circulating viruses in Russia.

In the 2019–2020 season, the Reference Center received mainly influenza A(H1N1)pdm09 and B viruses: 46.8% of A(H1N1)pdm09, 8.7% of influenza A(H3N2), and 44.5% of B/Victoria. According to the WHO National Influenza Center, all three influenza subtypes were present in the Russian population this season, with influenza B/Victoria virus predominating (43.7%) [22]. According to the European Center for Disease Prevention and Control (ECDC), in the European region, among the viruses that were typed, 51% A(H1N1)pdm09, 40.1% A(H3N2), 8.7% B/Victoria, 0.2% B/Yamagata were detected[3]. A(H3N2) viruses were the second most abundant, but not B/Victoria influenza vi-

[3] ECDC. Influenza virus characterisation, summary Europe, July 2020. Stockholm; 2020. URL: https://www.ecdc.europa.eu/en/publications-data/influenza-virus-characterisation-summary-europe-july-2020

ruses as in Russia, which may be due to the different patterns of spread of these virus types in different countries.

In the 2020–2021 season, only 2 influenza viruses (A(H3N2)) were tested in the Reference Center. According to the WHO National Influenza Centre, in Russia, only 37 positive materials were found during the entire season during PCR-based influenza screening, and only 2 influenza B/Victoria viruses were isolated in culture and antigenically characterized [22]. In the European region, this season also saw a 99.4% decrease in the number of confirmed influenza cases compared to the 2019–2020 season [22]. Influenza viruses were distributed as follows: 14.2% A(H1N1)pdm09, 80.6% A(H3N2), 4.9% B/Victoria, 0.3% B/Yamagata [9], i.e., just as in Russia, A(H3N2) virus predominated.

The low activity of influenza viruses worldwide was caused by the emergence of the novel betacoronavirus SARS-CoV-2 in December 2019 in China and subsequent preventive and protective measures [23]. These included restrictions on the movement of people: closing countries' borders, suspending international flights, quarantining those arriving in the country and isolating those who became ill. In addition, personal hygiene practices (frequent hand washing, use of disinfectants and personal protective equipment) were monitored to reduce transmission of influenza viruses. The timing of these measures correlates directly with the sharp drop in influenza incidence in 2020–2021.

In the 2021–2022 season, the number of viruses tested at the Reference Center increased significantly, with influenza A(H3N2) virus predominating (94.7%,) and influenza B viruses accounting for 5.3%. No samples containing influenza A(H1N1)pdm09 virus were received. According to the WHO National Influenza Center, the A(H3N2) subtype was predominantly detected in Russia, with minor involvement of influenza B/Victoria and complete absence of A(H1N1)pdm09 virus [21]. Thus, the etiologic structure of influenza this season in Russia was represented by influenza A(H3N2) virus with a minor contribution of influenza B virus.

According to ECDC data, influenza A(H3N2) virus was also predominant in the European region, with A(H1N1)pdm09 (8.7%) and B being detected much less frequently: A(H3N2) 90.9%, B/Victoria 0.4%, B-Yamagata < 0.1%[4]. It should be noted that despite reports of isolated findings of B/Yamagata influenza viruses, available databases of genetic information do not contain the HA nucleotide sequences of B/Yamagata influenza viruses detected after March 2020, which means that their affiliation with the Yamagata lineage has not been confirmed by sequencing.

The spectrum of influenza viruses examined in the 2022–2023 season at the Reference Center was as follows: 75.5% influenza A(H1N1)pdm09 viruses, 2.1% influenza A(H3N2) viruses, 22.4% influenza B/Victoria viruses. Among the viruses admitted and examined, 12%, predominantly influenza A(H1N1)pdm09 and to a lesser extent influenza B/Victoria, were viruses found in autopsy material, indicating an increase in influenza severity compared with the 2021–2022 season, when influenza A(H3N2) virus predominated and no autopsy material was admitted (Table 1). The increased number of influenza deaths during seasons of active circulation of influenza A(H1N1)pdm09 virus can be explained by the greater affinity of this influenza A virus subtype for SA-α2,3, which facilitates its spread to the lungs and leads to the development of pneumonia more often. In addition, a lower level of immunity in the population due to lack of exposure to influenza A(H1N1)pdm09 virus during the 2020–2021 and 2021–2022 seasons could have contributed to a more severe epidemic.

According to the WHO National Influenza Center, in Russia as a whole, at the peak of the influenza and acute respiratory viral illness epidemic, influenza viruses were detected in 30% of patient samples examined during PCR screening, with the A(H1N1)pdm09 subtype predominating[5]. In the 2022–2023 season, according to ECDC data, the structure of influenza in the European region differed from that in Russia: 47.3% — A(H1N1) pdm09, 47.9% — A(H3N2), 4.8% — B/Victoria, 0% — B/Yamagata[6], which could be due to the various epidemiological features of the spread of these types of viruses in different countries.

Our data on the distribution of A(H1N1)pdm09 viruses by genetic groups coincided with the data of the A.A. Smorodintsev Research Institute of Influenza: in the 2019–2020 season, the genetic subgroup 6B.1A5 (reference strain A/Norway/3433/2018) prevailed[7], in the 2022–2023 season, the genetic subgroup 6B.1A.5a.2 (reference virus A/Sydney/5/2021) prevailed[8].

The significance of the detected mutations leading to amino acid substitutions was assessed according to the literature data confirmed experimentally. It should

[4] ECDC. Influenza virus characterisation, Summary Europe, July 2021. Stockholm; 2021. URL: https://www.ecdc.europa.eu/en/publications-data/influenza-virus-characterisation-summary-europe-july-2021

[5] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2023. URL: https://influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2023&week=39

[6] ECDC. Influenza virus characterization: summary report, Europe, February 2023. Copenhagen–Stockholm; 2023. URL: https://www.ecdc.europa.eu/en/publications-data/influenza-virus-characterization-summary-europe-february-2023

[7] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2020. URL: https://www.influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2020&week=39

[8] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2023. URL: https://influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2023&week=39

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be taken into account that different amino acids differ in physicochemical properties (nonpolar, polar charged and uncharged), they can change the spatial configuration of the protein in different ways, even if the substitution occurred in the same position of the amino acid chain. The functional significance of each mutation must be proven experimentally. In some cases, it is acceptable to draw an analogy, for example, between HAs of subtypes H1 and H5, because HAs of influenza A H1 and H5 viruses belong to the same group (H1/H2/H5/H6/H11/H13), which, according to the predicted amino acid sequence of HAs, may have similar HA spatial conformational structures [25].

N156K and *L161I* mutations were detected in the antigenic Sa site of influenza A(H1N1)pdm09 virus, both in 1 virus. The *S159N* mutation in the HA of influenza A(H5N1) virus (position equivalent to *N156K* in the A(H1N1)pdm09 influenza viruses examined) results in enhanced binding to α-2,6-SA of the ferret respiratory tract mucosa [26]. For the *N156K* mutation in A(H1N1)pdm09, a change in antigenic properties was predicted in ferret experiments [27].

T185I, *D187A*, *Q189E*, and *S190N* mutations were found in the antigenic site Sb of influenza A(H1N1) pdm09 virus. In HA of influenza A(H1N1) virus, the *D187A* substitution (190 by H3 numbering) results in decreased binding to α-2,6- and increased affinity for α-2,3-SA [28]. In HA of epidemic influenza A(H1N1) virus, substitution of *S190N* (193 by H3 numbering) changes the conformation of the Sb antigenic site [29]. An *R205K* mutation was found near the Ca1 antigenic site of A(H1N1)pdm09 virus. In HA of H5N1 virus, substitution of *N224K* (numbered H3) at an equivalent position leads to enhanced binding to α-2,6-SA [30].

Near the antigenic Ca2 site of A(H1N1)pdm09 virus, the *R221K* mutation was found to be part of the HA A(H1N1)pdm09 receptor-binding site. It was shown that changes in this HA can affect the antigenic properties of viruses [31].

The amino acid arginine (R) at position 223 enhances the affinity of A(H1N1)pdm09 for avian-type receptors (α-2,3-SAs). Given the fact that α-2,3-SAs are part of the glycocalyx of the epithelial cells lining human lung alveoli, such mutant strains can cause pneumonia, and further generalized inflammation. By 2020, the majority of circulating influenza A(H1N1)pdm09 viruses (99.80%) had the *R223Q* mutation, including all the viruses we sequenced, whereas at the early stage of the 2009 pandemic, strains with an arginine (R) in this position were still circulating in a minor population [32]. Apparently, the elimination of mutations that may reduce the spread of viruses due to the high danger of such strains for host life is one of the mechanisms of influenza viruses evolution.

Outside the antigenic sites, 2 viruses of the A(H1N1)pdm09 subtype had the *D94N* mutation and 1 had the *D94E* mutation. In HA of influenza A(H5N1) virus, the *D94N* substitution results in decreased binding to α-2,6-SA and increased affinity for α-2,3-SA, and enhances HA-mediated fusion to the membrane of mammalian cells [33].

The *E224A* mutation in the receptor-binding site of A(H1N1)pdm09 increases the affinity for α-2,3-SAs (bird-type receptors) localized in human lungs [34].

The *D222N* mutation in the receptor-binding site also enhances binding to α-2,3-SA. The *D222N* and *D222G* mutations are associated with a severe course of influenza, including pneumonia and acute respiratory distress syndrome [35]. Our earlier molecular genetic analysis of influenza A(H1N1)pdm09 viruses circulating in Russia from 2009 to 2014 showed that amino acid substitution of D for G or N at position 222 was statistically significantly more frequently found in the lungs of deceased patients than in respiratory swabs of recovered patients ($p < 0.0001$ and $p = 0.007$) [36]. In the 2019–2020 season, the *D222N* mutation was detected in 1 (2%) virus from bronchoalveolar lavage of a patient with severe community-acquired pneumonia. In the 2022–2023 season, the *D222N* mutation was present in influenza A(H1N1)pdm09 viruses found in autopsy specimens from 12 patients (22% of viruses from autopsy material; 3% of total).

Our data on the distribution of A(H3N2) viruses by genetic groups matched the data of the Smorodintsev Research Institute of Influenza. In the 2019–2020 season, viruses of genetic subgroups 3C.2 a1b + *T131K* (reference strain A/South Australia/34/2019) and 3C.2a1b + T135K-B (reference strain A/Hong Kong/2675/2019)[9] circulated in the ratio of 2 : 1; in 2021–2022, the genetic subgroup 3C.2a1b.2a.2 prevailed[10], and in 2022–2023 — subgroup 3C.2a1b.2a.2 (reference virus Bangladesh/4005/2020)[11].

In the 2020–2021 season, no influenza A(H3N2) viruses were isolated at the Smorodintsev Influenza Research Institute[12], and the viruses we examined belonged to group 3C.2a1b.2a.2, which was consistent with the ECDC data: influenza A(H3N2) viruses circulating in the influenza A(H3N2) virus population were group 3C.2a1b viruses, most of which were Cambodia-

[9] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2020. URL: https://www.influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2020&week=39

[10] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 24th week of 2022. URL: https://www.influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2022&week=24

[11] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2023. URL: https://influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2023&week=39

[12] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2021. URL: https://www.influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2021&week=39

(3C.2a1b.2a.1) and Bangladesh-like (3C.2a1b.2a.2) viruses[13].

From 1968 to 2003, antigenic drift of influenza A(H3N2) virus was mainly caused by single mutations in 7 amino acid positions in HA (145 in site A, 155, 156, 158, 159, 189, 193 in site B) near the receptor-binding site [37].

In HA of influenza A(H3N2) virus, substitution *A131D* (*T131K* in our samples) increases the charge of the HA molecule and results in decreased neutralization by a monoclonal antibody [38]. The *S193R* and *S193K* mutations (*S193F* in our samples) have been shown to affect the preferential binding of the virus to α2,6 and α2,3 SAs, respectively [39].

In the HA of swine influenza A(H3N2) virus, the S138A substitution (similar to that found in the samples we examined) leads to reduced virus replication in swine respiratory tract epithelial cells that express α2,6 and α2,3 SA receptors [40].

In HA of influenza A(H3N2) virus, substitution *K156Q* (*H156S* in our samples) leads to a decrease in the neutralizing antibody activity. This is due to the fact that this amino acid molds the globular head of HA, where it forms a new epitope adjacent to the receptor-binding domain [30].

The data we obtained on the distribution of influenza B viruses by genetic groups also corresponded to the data of the Smorodintsev Research Institute of Influenza: in 2019–2020, the absolute majority of sequenced influenza B viruses belonged to the V1A (del162-164) clade of the Victorian lineage (reference virus B/Washington/02/2019)[14]; in the 2021–2022 season, influenza B/Victoria viruses of the genetic subgroup V1A.3 a.2[15] were found, in the 2022–2023 season, influenza type B viruses were assigned to genetic subgroup V1A.3a.2 and similar to the reference virus B/Austria/1359417/2021.

A D197E mutation in the HA receptor-binding site was identified in 40 viruses. The significance of amino acid substitutions in this position of HA was experimentally proved by a group of researchers by passaging strain B/Brisbane/60/2008 in human lung epithelial cell line Calu-3: after 10 consecutive passages, mutation D197T appeared in HA and it was shown that strains with this substitution had significantly lower affinity to SA-α2,3 (bird type). This could be explained by the fact that α2,3-linked glycan forms 2 hydrogen bonds with the amino acid at position 197, and any substitution at this position could affect the binding of HA to receptors [41].

Conclusion

The article presents the results of genetic monitoring of influenza viruses A(H1N1)pdm09, A(H3N2) and B detected in 50 regions of Russia from 2019 to 2023. The results reflect general patterns and allow us to judge the etiologic structure of influenza, the intensity of the epidemic process and the genetic diversity of viruses circulating in Russia.

In the Rospotrebnadzor system, influenza viruses are monitored annually; even in the 2020–2021 season, against the background of the COVID-19 pandemic, screening of patients for influenza by PCR with hybridization-fluorescence detection of amplification products, typing and sequencing of detected influenza viruses continued in the same volume. That is why it can be stated that in Russia against the background of the COVID-19 pandemic in the season 2020–2021 influenza viruses practically disappeared from circulation and appeared again in the season 2021–2022.

The analysis demonstrated the phenomena of continuous evolution with the appearance in each season of genetic variants of influenza viruses A(H1N1)pdm09, A(H3N2) and B that had changes in the HA gene compared to the vaccine strain. Mutations leading to HA amino acid substitutions were recorded in antigenic sites, in the receptor binding region, some of them leading to the formation of new potential glycosylation sites or to their loss.

When comparing influenza A(H1N1)pdm09 viruses circulating in 2022–2023 in Russia with the 2019–2020 vaccine strain A/Brisbane/02/2018, the degree of differences in the nucleotide sequences of the HA gene was 2.7–3.1%, and with the first vaccine strain of influenza A(H1N1)pdm09 virus A/California/07/2009 — 5.0–5.3%.

The degree of difference in the nucleotide sequences of the HA gene of influenza A(H3N2) viruses circulating in 2022–2023 in Russia with the 2019–2020 vaccine strain A/Kansas/14/2017 amounted to 5.3–6.0%.

The level of difference in the nucleotide sequences of the HA gene of influenza B/Victoria viruses circulating in 2022–2023 in Russia with the 2019–2020 vaccine strain B/Colorado/06/2017 was 2.2–2.8%. Influenza B/Yamagata viruses were not identified during the study period.

The highest HA variability was observed for A(H3N2) viruses, which necessitated changing the vaccine strain 3 times in 4 seasons.

Of particular note, all influenza A(H1N1)pdm09 viruses from the 2022–2023 season had the previously unknown *E224A* mutation in HA, which increases affinity for SA-α2,3 localized in the human lung, which may contribute to complications. The *D222N* mutation,

[13] ECDC. Influenza virus characterisation, Summary Europe, July 2021. Stockholm; 2021. URL: https://www.ecdc.europa.eu/en/publications-data/influenza-virus-characterisation-summary-europe-july-2021

[14] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 39th week of 2020. URL: https://www.influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2020&week=39

[15] A.A. Smorodintsev Research Institute of Influenza. Weekly national influenza and ARVI bulletin for the 24th week of 2022. URL: https://www.influenza.spb.ru/system/epidemic_situation/laboratory_diagnostics/?year=2022&week=24

which is associated with more severe disease, was found in HA in 2% and 3% of influenza A(H1N1)pdm09 viruses in 2019–2020 and 2022–2023, respectively.

Almost all influenza viruses were sensitive to oseltamivir and zanamivir, only 2.3% of influenza A(H1N1)pdm09 viruses in 2022–2023 showed resistance mutation *H275Y* in NA. In all influenza A(H1N1) pdm09 and A(H3N2) viruses studied, an adamantane resistance mutation *S31N* in M2 was found.

Our results may help to understand the direction of evolution of influenza viruses. The continuous emergence of mutations in influenza viruses poses a global public health challenge because some mutations provide a selective advantage for viral replication in the upper respiratory tract and human-to-human transmission, and reduce sensitivity to antiviral drugs. Some mutations contribute to a more severe course of influenza and the development of complications. Mutations in antigenic sites allow influenza viruses to evade anamnestic and vaccine-induced antibodies.

Therefore, it is necessary to continue to monitor influenza viruses using molecular genetic analysis, which allows deep differentiation of influenza viruses and determines the trend in the evolution of influenza viruses: the emergence, spread and disappearance from circulation of certain genetic variants.

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Оригинальное исследование
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Разработка и исследование вируснейтрализующей активности рекомбинантного человеческого антитела к F-гликопротеину респираторно-синцитиального вируса

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Аннотация

Введение. Респираторно-синцитиальный вирус (РСВ) является ведущим в структуре возбудителей инфекций нижних дыхательных путей у детей, а также представляет серьёзную угрозу для пожилых людей и пациентов с ослабленным иммунитетом. Разработка терапевтического препарата на основе рекомбинантных человеческих антител, направленных на блокирование F-гликопротеина РСВ, является актуальной задачей, поскольку позволит снизить заболеваемость РСВ-инфекцией и предотвратит развитие осложнений данной инфекции.

Цель исследования — конструирование плазмидных векторов для накопления высокоактивного рекомбинантного моноклонального антитела FM1 в эукариотической системе экспрессии, направленного против F-гликопротеина РСВ, и оценка специфической активности полученного антитела в отношении различных штаммов РСВ подтипов А и В *in vitro*.

Материалы и методы. Получение экспрессионных конструкций, кодирующих рекомбинантное антитело FM1, выполняли методами генной инженерии. Накопление антитела проводили в клеточной линии CHO-K1 путём транзиентной экспрессии. Препарат антитела очищали из культуральной жидкости методом аффинной хроматографии с использованием в качестве лиганда модифицированного белка А. Оценку вируснейтрализующей активности антитела оценивали в реакции микронейтрализации с несколькими штаммами РСВ на монослойной культуре клеток Vero.

Результаты. Создана двухплазмидная векторная система для экспрессии рекомбинантного антитела FM1 к F-гликопротеину РСВ, получен временный CHO-продуцент этого антитела. Антитело накоплено, очищено и охарактеризовано; доказана его биологическая активность. Продемонстрировано, что антитело обладает повышенной вируснейтрализующей активностью в отношении эталонных и сезонных штаммов РСВ подтипов А и В по сравнению с контрольным препаратом паливизумабом.

Заключение. Препарат на основе полученного рекомбинантного антитела FM1 позволит решить проблему импортозамещения средств защиты против РСВ-инфекции. В настоящее время коллектив авторов ведёт работу над получением стабильного клона-продуцента FM1 с высокой продуктивностью и жизнеспособностью, а также исследует терапевтическую эффективность этого антитела на модели сублетальной РСВ-инфекции у мышей.

Ключевые слова: *терапевтические моноклональные антитела, респираторно-синцитиальный вирус, F-гликопротеин, реакция нейтрализации, CHO-продуцент рекомбинантных антител, культура клеток Vero*

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Original Study Article
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Development and evaluation of a recombinant monoclonal human antibody with virus-neutralizing activity against the F glycoprotein of respiratory syncytial virus

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Abstract

Introduction. Respiratory syncytial virus (RSV) is the most common pathogen causing lower respiratory tract infections in children. RSV also poses a serious threat to the elderly and immunocompromised patients. Developing a therapy based on recombinant human antibodies to block the RSV fusion (F) glycoprotein is urgent to reduce the incidence of RSV infections and prevent associated complications.

Aim. To design plasmid vectors for efficient production of the recombinant monoclonal antibody FM1 in a eukaryotic expression system targeting the RSV fusion (F) glycoprotein and to evaluate its activity against RSV subtypes A and B in vitro.

Materials and methods. Constructs encoding the recombinant antibody FM1 were designed using genetic engineering. Recombinant antibodies were produced in the CHO-K1 cell line through transient expression. Antibody specimens were purified from the culture supernatant using affinity chromatography, with a modified protein A as the ligand. The virus-neutralizing activity of the antibody was evaluated in a microneutralization assay using several RSV strains on a Vero cell monolayer culture.

Results. We developed a two-plasmid vector system to produce the recombinant FM1 antibody targeting the RSV F glycoprotein, using CHO cells as transient producers. The antibody was successfully produced, purified, and characterized, with its biological activity confirmed. The FM1 antibody demonstrated enhanced virus-neutralizing activity against reference and seasonal RSV strains of subtypes A and B compared to the control drug palivizumab.

Conclusion. A recombinant FM1 antibody-based drug could address the import substitution challenge for protective measures against RSV infection. The authors are currently developing a stable FM1 producer clone with high productivity and viability and investigating the therapeutic efficacy of this antibody in a sublethal RSV infection mouse model.

Keywords: *therapeutic monoclonal antibodies, respiratory syncytial virus, fusion (F) glycoprotein, neutralization assay, CHO cell line for recombinant antibodies production, Vero cells*

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Introduction

Respiratory syncytial virus (RSV) is the most prominent pathogen of lower respiratory tract infections in children and also poses a serious threat to the elderly and immunocompromised patients [1, 2].

Up to 70% of children have their first RSV infection (RSVI) before the age of 1 year, and almost every child is infected during the first 3 years of life. The frequency of RSVI verification in children under 3 years of age hospitalized for lower respiratory tract infection reaches 42–63% in developed countries [3, 4]. Bronchiolitis is the most common (50–90%), pneumonia (5–40%) and tracheobronchitis (10–30%) are slightly less common, and mortality averages 1% [5–7]. According to the results of meta-analysis of morbidity in 132 developed countries, RSVI accounts for more than 3 million hospitalizations per year and about 60 thousand deaths among children under 5 years of age [8, 9].

According to the data of polymerase chain reaction diagnostics conducted at the A.A. Smorodintsev Research Institute of Influenza, in the season of 2023–2024, during the peak period of acute respiratory viral infections the share of RSV among respiratory pathogens amounted to 26% excluding SARS-CoV-2 and influenza viruses and 16% including influenza viruses, which clearly indicates a significant role of RSVI in the structure of respiratory infections, especially in children under 2 years of age. According to the data of the A.A. Smorodintsev Research Institute of Influenza, the share of RSVI among hospitalized patients is 13-19% [10]. Given that about 30 million patients with respiratory infections are registered annually in Russia, RSVI accounts for at least 3.9 million of these cases.

According to the estimates of T. Shi et al., 45% of hospitalizations and in-hospital deaths in children under 6 months of age are due to acute respiratory failure resulting from RSVI [9]. Since vaccines are less immunogenic at this age, maternal immunization or monoclonal antibody (MCA) administration can be used to induce passive immunity in infants to provide better protection for the child.

Antibodies are thought to play a key role in limiting acute lower respiratory tract infection in RSVI. Recent studies emphasize that induction of mucosal immunity is required for a full immune response to protect against reinfection [11]. Until recently, the only means of preventing RSVI was the humanized MCA palivizumab [12], which was used only in at-risk groups and required repeated injections. Currently, a new drug, nirsevimab, has been developed and approved in the EU and the USA, which is more stable and can be administered once [13–15]. In Russia, only palivizumab is registered for clinical use. The development of a preparation for the prevention and therapy of RSVI based on recombinant human neutralizing antibodies interacting with the surface glycoprotein F of RSVI will significantly reduce the incidence of RSVI in young children, reduce disability and mortality caused by this pathogen, and prevent the development of complications of this infection. There is also an urgent need for means of prevention and therapy of RSVI in the elderly and immunocompromised patients.

The aim of the present study was to construct plasmid vectors for the accumulation of highly active recombinant MCA (rMCA) FM1 in a eukaryotic expression system directed against the glycoprotein F of RSV and to evaluate the specific activity of the resulting antibody against different strains of RSV subtypes A and B *in vitro*.

Materials and methods

Construction of plasmid vectors

Nucleotide insertions encoding the heavy and light chains of rMCA FM1 (including constant sites) were assembled on the basis of published sequences of the MEDI8897 antibody [16] and synthesized at Eurogen. Cloning was performed into pVAX1 vector using restriction sites for Nhe I and Xho I endonucleases. Colonies were screened by polymerase chain reaction, the presence of target insertions was confirmed by Sanger sequencing at Eurogen, after which the created plasmid constructs pVAX1-FM1-HC and pVAX1-FM1-LC were accumulated, purified using the Plasmid Miniprep 2.0 kit (Eurogen) and used for transfection of eukaryotic cells.

DNA electrophoresis in agarose gel

Plasmid DNA preparations and amplicons were analyzed in 0.8% agarose gel prepared in 1× TAE buffer containing up to 0.5 μg/ml ethidium bromide using 6× DNA plating buffer. The results of electrophoretic separation were visualized using a Gel Doc EZ Imager (Bio-Rad).

Cells and viruses

CHO-K1 cells (Chinese hamster ovary cells, ATCC #CCL-61) and Vero cells (African green monkey kidney cells, ATCC #CCL-81) obtained from the ATCC cell culture bank (American Type Culture Collection) were used in the experiments. CHO-K1 cells were cultured on F12K medium (Gibco) supplemented with 10% FBS (Gibco), while Vero cells were cultured on α-MEM medium (Biolot) supplemented with 5% FBS (Gibco). All experiments (except for the stages of selection of CHO-K1 producers) were performed without antibiotics. Daily cultures were used in this study. All cell cultures were maintained at a temperature of 37.0 ± 0.5ºC, in an atmosphere of 5% CO_2, under high humidity conditions (80-100%).

We used RSV of two reference strains: A2 (subtype A), infectious titer 7.7 $lgTID_{50}$/mL and 9320 (subtype B), infectious titer 6.8 $lgTID_{50}$/mL; as well as 2 sea-

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sonal RSV isolates: hRSV/A/Russia/RII-26062v/2022 (subtype A), infectious titer 6.8 lgTID$_{50}$/mL and hRSV/B/Russia/RII-4759/2022 (subtype B), infectious titer 6.3 lgTID$_{50}$/mL (Collection of the A.A. Smorodintsev Research Institute of Influenza).

The commercial drug Synagis (AstraZeneca; solution for intramuscular administration, 100 mg/ml, series 406039, manufactured 08.2023, valid until 07.2026), which is a humanized MCA palivizumab directed against glycoprotein F of RSV, was used as a comparison preparation [17].

Enzyme immunoassay

Enzyme-linked immunosorbent assay (ELISA) in sandwich format was performed using 96-well Microlon High Binding plates (Greiner Bio-One), PST-60HL-4 thermoshaker (BioSan), commercial MCAs, control preparation of palivizumab, and recombinant antibodies and purified viruses obtained at the A.A. Smorodintsev Research Institute of Influenza. Capture antibodies against Fc-fragments of human immunoglobulin heavy chains (#ab77118, Abcam) were sorbed at a concentration of 1 μg/mL in a volume of 100 μl per well at 4ºC overnight. Blocking was performed with a solution of 5% skim milk (Blotting-Grade Blocker, #1706404, Bio-Rad) on PBST (Tween-20 to 0.05%) at 37ºC for 1 h. Incubation with the analyzed samples was performed at 37ºC for 2 h, after which detection antibodies against human immunoglobulin light kappa chains (#4G7cc, Hytest) conjugated to horseradish peroxidase were added at the manufacturer's recommended concentration and incubated at 37ºC for 1 h. After standard detection using a substrate mixture of tetramethylbenzidine (Chema) and mononormal sulfuric acid, optical density (OD) was measured at wavelengths of 450 nm (OD_{450}) and 655 nm (OD_{655}) on a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific). The average value of $OD_{450–655}$ for all negative controls plus 3 standard deviations was taken as the threshold value.

Chromatographic purification of recombinant antibodies

Chromatographic purification of recombinant antibodies was performed by affinity chromatography using an AKTA pure chromatography system on a 5 mL MabPurix P45 column (Sepax). The column was washed with 10 CV (column volume) of starting buffer (1× PBS) at a flow rate of 5 mL/min. The culture liquid (50 mL), pre-filtered through a Sartorius syringe filter (pore size 0.45 μm, polyethersulfone membrane material), was introduced into the chromatograph through a sample pump at a flow rate of 2.5 mL/min. The column was then washed with 10 CV of starting buffer at a flow rate of 5 mL/min. Antibodies were eluted with 100% elution buffer (20 mM glycine, pH 3.0) in a volume of 15 CV at a flow rate of 5 mL/min. Monitoring was performed using OD_{280}. During the elution step, peaks with OD above 0.05 AU were selected using an automated collector. To the collected material, 1 M Tris-HCl pH 8.8 solution (20 μL/mL) and 4 M NaCl solution (40 μL/mL) were added. To increase sterility and prevent degradation and contamination, the obtained preparation was filtered using a Sartorius syringe filter (pore size 0.45 μm, polyethersulfone membrane material) and used for further studies.

Protein electrophoresis in polyacrylamide gel

Polyacrylamide gel (PAAG) electrophoresis was performed according to the Lammlie method [18] under reducing (in the presence of β-mercaptoethanol) and non-reducing conditions. A 15-well Any kD gradient gel (#4568126, Bio-Rad) was used. The sample was mixed with 4-fold Lammlie's buffer before being applied to the PAAG wells, followed by protein denaturation at 95ºC in a Gnome solid-state thermostat (DNA-Technology) for 10 min (reducing conditions). 2.5 μg of protein sample was added to each well. Protein concentration was evaluated on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and for antibodies the IgG mode was used, with an E value of 1% = 13.70. Electrophoretic separation of proteins was performed at constant current (25 mA per gel) for 45 min in a Mini-PROTEAN Tetra vertical electrophoretic cell (Bio-Rad). The gel was stained with Coomassie colloidal solution [19]. The stained gel was imaged on a Gel Doc EZ Imager gel documentation station (Bio-Rad).

Microneutralization reaction and determination of half-maximal inhibitory dose

The virus-neutralizing activity of recombinant antibodies was evaluated on Vero monolayer cell culture using the method described previously [20]. A series of triplicate dilutions of recombinant antibody preparations (3 independent repeats) were mixed with an equivalent volume of growth medium containing 100 TID_{50} RSV, and after 1 h incubation at room temperature, the dilutions were transferred to plates with a daily monolayer of Vero cells. The plates were incubated for 4 days in a CO_2 incubator at 37.0 ± 0.5ºC under high humidity conditions (80–100%). Inhibition of RSV replication in the presence of different concentrations of rMCA was determined on the 4th day after infection by cell ELISA using as primary mouse antibody MCA 4F2 specific to RSV F-glycoprotein of subtypes A and B (A.A. Smorodintsev Research Institute of Influenza) and horseradish peroxidase-labeled goat anti-mouse IgG (H+L) secondary antibody (Bio-Rad). After conjugate-substrate color reaction, the OD was measured using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific) and calculated as the difference of $OD_{450–620}$. The obtained OD values

were transformed into the percentage of inhibition of the development of cytopathic action of the virus at a certain concentration of recombinant antibodies. The half-maximal inhibitory concentration (IC_{50}) was calculated by plotting a four-parameter dose-effect curve using GraphPad Prism 9.5.1 software on the basis of 3 independent repeats.

Primary data and statistical processing

Statistical analysis of primary data was performed in the Microsoft Office Excel 2010 and GraphPad Prism 9.5.1 software packages. The following statistical indicators were used to present the data: standard deviation, arithmetic mean, standard error of the mean. The Shapiro–Wilk test was used to test the hypothesis of normality of the obtained distribution of values, and Student's *t*-test was used to determine the significance of differences between group averages. The a priori level of significance was taken as $\alpha = 0.05$. Differences were considered reliable at the achieved significance level $p < \alpha$ [21].

Results

Design and generation of expression constructs encoding recombinant FM1 antibody

The sequences of the MEDI8897 antibody were chosen as the basis for the design of a long-acting human rMCA FM1 for the prevention of lower respiratory tract diseases caused by RSVI [16].

The antibody under development is a recombinant human immunoglobulin of the IgG1κ class. MEDI8897 has the following sequences of hypervariable regions: in the light chain, L1 QASQDIVNYLN, L2 VASNLET and L3 QQYDNLPLT; in the heavy chain, H1 DYIIN, H2 GIIPVLGTVHYGPKFQG and H3 VSETYLPHYFDN [16]. The constant region of the MEDI8897 light chain belongs to the human κ-isotype (encoded by the κ locus 2p11.2 on the 2nd chromosome) and is completely identical to the canonical sequence P01834 presented in the UniProt open protein sequence database [22]. The constant sequence of the heavy chain MEDI8897 belongs to the immunoglobulin G1 class (sIgG1, secreted form) and has several differences from the sequence P01857 presented in UniProt. In particular, in addition to the intentionally introduced 3 amino acid substitutions in the CH2 domain of the constant region (M257Y/S259T/T261E, [YTE]) in MEDI8897 that ensure prolonged circulation of the antibody in the blood [16], we also detected 2 substitutions in the constant regions of MEDI8897: K97R (VAR_003886) and D239E (VAR_003887), which are variant natural substitutions in the alleles [22]. Thus, despite the presence of variation, the antibody amino acid sequences that fully match the heavy and light chain sequences of MEDI8897 were selected for cloning [16].

A 2-plasmid expression system was chosen for FM1 rMCA production, which implies the presence of 2 vector constructs, one of which encodes the heavy and the other the light full-length chains of the antibody, i. e. the chains in the constructs contain both variable and constant regions of the antibody, as well as signal peptides.

The pVAX1 vector containing CMV promoter, T7-promoter at the 5′-end of the insert and polyadenylation site (from bovine growth hormone) was selected for cloning; selective antibiotics for the vector were: in bacterial system — ampicillin, in eukaryotic system — neomycin (kanamycin).

The pVAX1-based pVAX1-FM1-HC (total length 4349 bp) and pVAX1-FM1-LC (3632 bp) constructs capable of expression and production of FM1 rMCA in eukaryotic cells in the format of a full-length IgG1κ heterotetramer were assembled by genetic engineering methods. Schematic representations of the resulting constructs are shown in **Fig. 1**. Both sequences, the heavy chain (total length 1428 bp, variable part size 378 bp) and the light chain (total length 711 bp, variable part size 321 bp), contain the Kozak sequence, N-terminal leader peptides that ensure secretion of the full-length antibody, and are flanked by restriction sites Nhe I (at the 5′-end) and Xho I (at the 3′-end).

The developed plasmid constructs are capable of constitutive expression in eukaryotic cells (due to the presence of the CMV promoter) of mature polyadenylated mRNAs encoding the heavy and light chains of rMCA FM1.

Production of stable eukaryotic pools of recombinant FM1 antibody by temporary transfection

FM1 rMCA production was carried out by temporary transfection of eukaryotic cell line CHO-K1 with a 2-plasmid system providing the correct conformation and correct glycosylation of the formed antibody. To accumulate FM1 rMCA, we chose the monoselection variant, in which CHO-K1 cells were transfected with plasmid constructs based on a single pVAX1 vector. For this purpose, the obtained constructs pVAX1-FM1-HC and pVAX1-FM1-LC and commercial reagent Lipofectamine 3000 (Thermo Fisher Scientific) were used. The antibiotic geneticin (an analog of neomycin) in the concentration range of 100-400 μg/mL (for selection of clones carrying the NeoR/KanR resistance gene) was used as a selective agent for progenitor cells obtained using the CHO-K1 line.

To increase the probability of obtaining a greater number of antibody-producing clones, adaptation of the pool of transfected cells to selective conditions was performed before each cloning. The adaptation process consisted of passaging cells on selective medium every 3–4 days. At this time, the cells deprived of the genetic construct, including the selective marker gene, died. After several passages, the viability of cells in the pool

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was restored due to an increase in the growth rate of cells adapted to the selective medium.

According to the results of sandwich ELISA (using lower antibodies against Fc-fragment of heavy chains and upper antibodies against light κ-chains of human immunoglobulins, allowing to detect only full-size IgG1κ heterotetramers) transfected CHO-K1 cells were capable of stable production of FM1 antibody. The dynamics of rMCA accumulation was assessed during the first 6 days, thereafter the cells formed a 100% monolayer, the production of the target product by the cells reached a constant level and directly correlated with the number of cells. The concentration of FM1 rMCA in supernatants obtained from CHO-K1 cells during transient expression was measured by sandwich ELISA and was 10 μg/mL. After 15 days of culturing, it was possible to obtain populations capable of stable propagation under selection conditions and producing FM1 rMCA. Further, the obtained temporary CHO-producers were used for accumulation of preparative amount of rMCA FM1 and its subsequent chromatographic purification.

Accumulation, purification, integrity analysis and evaluation of specific activity of recombinant FM1 antibody

FM1 rMCA was purified from the culture fluid by affinity chromatography using modified protein A (MabPurix P45, Sepax) as a ligand. Supernatant from CHO-K1 cells was collected for 1 month every 5 days. Purification was performed from approximately 300 ml of cell supernatant to yield 2.8 mg of FM1 rMCA preparation for studies of its specific and virus-neutralizing activity.

The chromatogram of the purification of the original preparation by affinity chromatography is shown in **Fig. 2**, *a*. The concentration of purified FM1 preparation was about 0.7 mg/mL, and the total amount of preparation was about 3.5 ml. The drug integrity analysis was confirmed by electrophoresis in PAGE according to the Lammlie method [18] (**Fig. 2**, *b*).

rMCAs consist of 4 polypeptide chains: 2 heavy and 2 light chains connected into a heterotetramer by disulfide bonds. On the electrophoregram under nonreducing conditions the antibody has a molecular weight of about 150 kDa, under reducing conditions the tracks show major fragments with molecular weights of 50–60 and 25–30 kDa, which correspond to the heavy and light chains of rMCA. The purified FM1 preparation was shown to contain mainly recombinant immunoglobulins without visible impurities.

To evaluate the specific activity of FM1 preparation, namely the ability to bind target antigens, ELISA was performed in 2 variants: by sorption of formalin-inactivated purified RSV on a substrate, as well as in-cell ELISA – by infection of Vero cells with RSV A2 and RSV B 9320 strains at different doses. ELISA results showed that the resulting FM1 preparation specifically binds purified RSV at concentrations comparable to the control preparation palivizumab (data not shown).

Evaluation of virus neutralizing activity of recombinant FM1 antibody

To study the biological activity of the obtained FM1 preparation, its ability to neutralize infectious RSV of different subtypes *in vitro* was evaluated. The microneutralization reaction was performed on Vero cell culture against reference strains of RSV A2 and RSV B 9320, as well as seasonal RSV strains isolated in St. Petersburg: hRSV/A/Russia/RII-26062v/2022 (RSV A) and hRSV/B/Russia/RII-4759/2022 (RSV B). Detection of the degree of inhibition of cytopathic action of RSV strains was evaluated by ELISA method

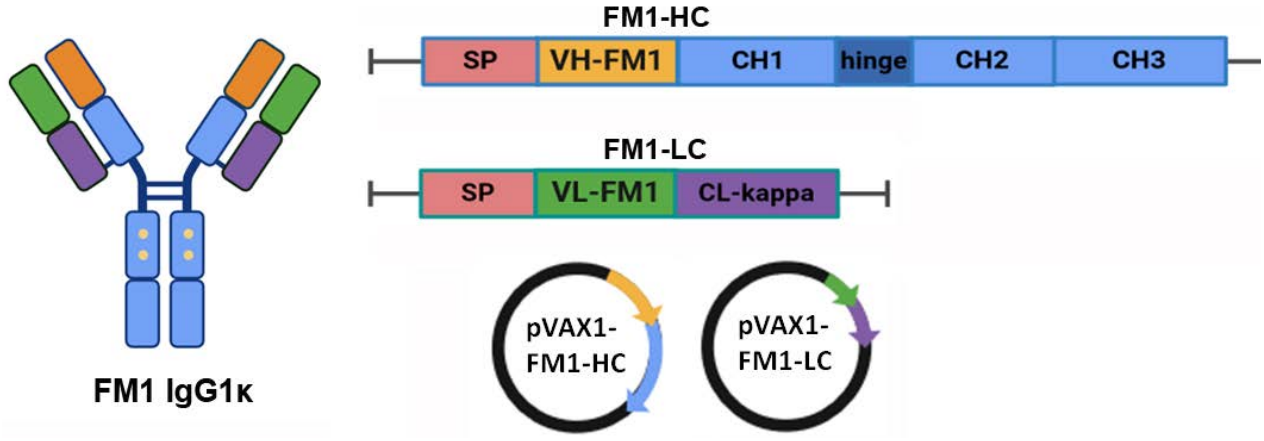

Fig. 1. Design and production of expression constructs encoding the recombinant antibody FM1 in the format of a full-size IgG1κ heterotetramer.

FM1-HC — heavy chain, FM1-LC — light chain, SP — signaling peptides, VH-FM1 — variable domain of heavy chain, CH1, CH2 and CH3 — constant domains of heavy chain, hinge — hinge section, VL-FM1 — variable domain of light chain, CL-kappa — constant domain of the light κ-chain.

with subsequent transformation of $OD_{450–655}$ values into the percentage of neutralization at a certain concentration of recombinant antibodies. Based on the results of the dose-effect curve plotting, a 50% inhibitory concentration (IC_{50}) was calculated against each tested strain based on 3 independent repeats (**Fig. 3**). Palivizumab was used as a comparison drug.

FM1 showed neutralizing activity against reference and seasonal strains of both RSV A and RSV B. The IC_{50} values for FM1 were significantly lower than those of the reference drug (Student's t-test, $p < 0.05$) (**Table**).

Discussion

RSV is the most prominent pathogen of severe pneumonia in children, requiring hospitalization, and also poses a serious threat to the elderly and immunocompromised patients. Currently, no antiviral drugs are registered for etiotropic therapy of RSVI. In Russia, an MCA-based drug, palivizumab, has been approved for RSVI prophylaxis in children, but its use has a number of clinical and economic limitations.

In 2023, for the first time in the last 20 years, three immunobiologic drugs were approved worldwide for RSVI prevention: a vaccine for people over 60 years of age, a vaccine for pregnant women, and nirsevimab, a drug based on human rMCAs. Nirsevimab has expanded indications for use and is recommended for all newborns during the first season of RSV circulation and for high-risk children under 2 years of age during the 2nd season [23, 24].

The main antigenic target for the development of prophylactic and therapeutic agents against RSVI is the RSV surface F-glycoprotein stabilized in the pre-fusion conformation, because antibodies to such an antigen have high virus-neutralizing activity [25]. The sequence of this glycoprotein is highly conserved among different RSV subtypes and genotypes. A decrease in the activity of RSV protein F prevents virus fusion with the cell, disrupts the mechanism of its penetration and protects the host from infection [26, 27]. Thus, obtaining antibody-based preparations aimed at blocking RSV glycoprotein F in the pre-fusion conformation is an urgent task.

The antibody MEDI8897 (nirsevimab) [28, 29] is a human rMCA of the IgG1κ class capable of high-affinity binding of the conserved spatial epitope formed by the F1 and F2 subunits of RSV glycoprotein F in the pre-fusion conformation (site Ø, a. o. 62–69 for F2 and 196–212 for F1) [30]. This binding interferes with the conformational mobility of the glycoprotein F required for fusion of the viral particle and cell membranes mediated by this protein, thus the MEDI8897 antibody blocks the fusion process and prevents virus entry into the host cell. The Fc-fragment of the MEDI8897 antibody has 3 amino acid substitutions, the presence of which significantly increases the circulation time of the antibody in the bloodstream. Thus,

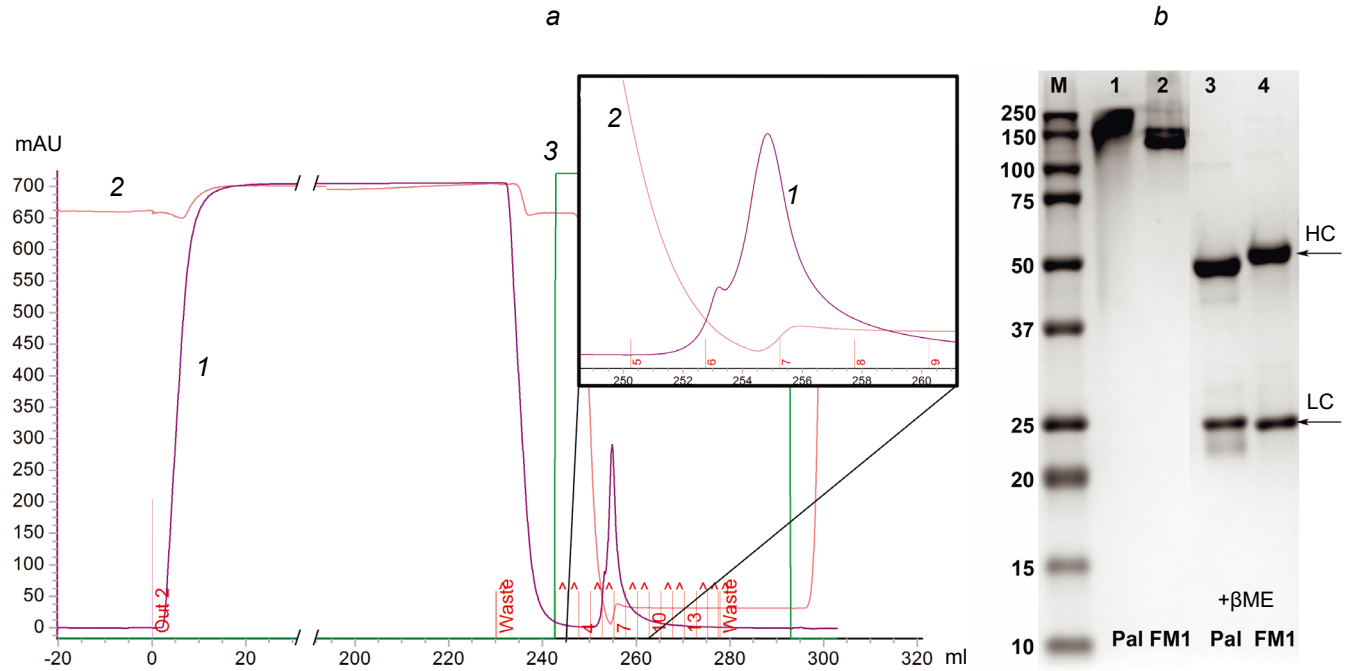

Fig. 2. Purification and analysis of the recombinant antibody FM1 specimen.

a — chromatogram of the purified FM1 preparation (the peak of elution is framed, enlarged image). *1* — absorption at a wavelength of 280 nm (MAU); *2* — conductivity (mSm/cm); *3* — elution (% of the elution buffer).

b — the results of the electrophoresis in PAAG of the purified FM1 drug compared with the control drug palivizumab (Pal). The preparations were applied in non-reduced (tracks 1 and 2) and reduced conditions (tracks 3 and 4; βME — β-mercaptoethanol). M — molecular weight marker (Bio-Rad). On tracks 3 and 4 there are visible zones corresponding to electrophoretic mobility of recombinant antibody heavy (HC)and light (LC) chains. The gel was colored by Coomassie colloidal solution and processing using the Gel Doc EZ Imager (Bio-Rad).

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a single intramuscular injection of MEDI8897 can protect the organism during one epidemic season of RSVI (i.e., about 150 days after administration) [13, 14]. This antibody has a neutralizing effect against human RSV strains of antigenic subtypes A and B circulating simultaneously in local epidemics and is intended for the prevention of lower respiratory tract diseases caused by RSV infection [30].

In our study, we obtained and characterized human rMCA FM1, the design of which was based on the sequences of heavy and light chains of the antibody MEDI8897 [16]. Additionally, sequences of signaling peptides were selected to ensure secretion of the full-length antibody for its efficient accumulation in the extracellular space.

rMCA, like many other proteins, is secreted by cells via the co-translational translocation pathway. In eukaryotes, a signal peptide containing 5–30 amino acid residues that are present at the N-terminus of expressed proteins is recognized by a signal recognition particle in the cytosol while still in the process of synthesis on ribosomes, and after passing through the endoplasmic reticulum, the signal peptide is cleaved off by a signal peptidase. Efficient expression of heavy and light chains requires appropriate signal peptides to transport the polypeptide chains of the antibody into the endoplasmic reticulum for proper folding, assembly, and posttranslational modification.

Since there was no information in the literature on the sequences encoding signal peptides in the heavy and light chain constructs of MEDI8897, we chose the combination of H7/L1 signal peptides according to the study conducted by R. Haryadi et al. [31], in which 8 heavy chain signal peptides and 2 light chain signal peptides were analyzed for their effect on the level of production in CHO cells of 5 most commercially successful therapeutic rMCAs. This study shows that the best signaling peptide for the heavy chain of most of the antibodies tested (adalimumab, bevacizumab, infliximab) was the H7 sequence. When choosing between

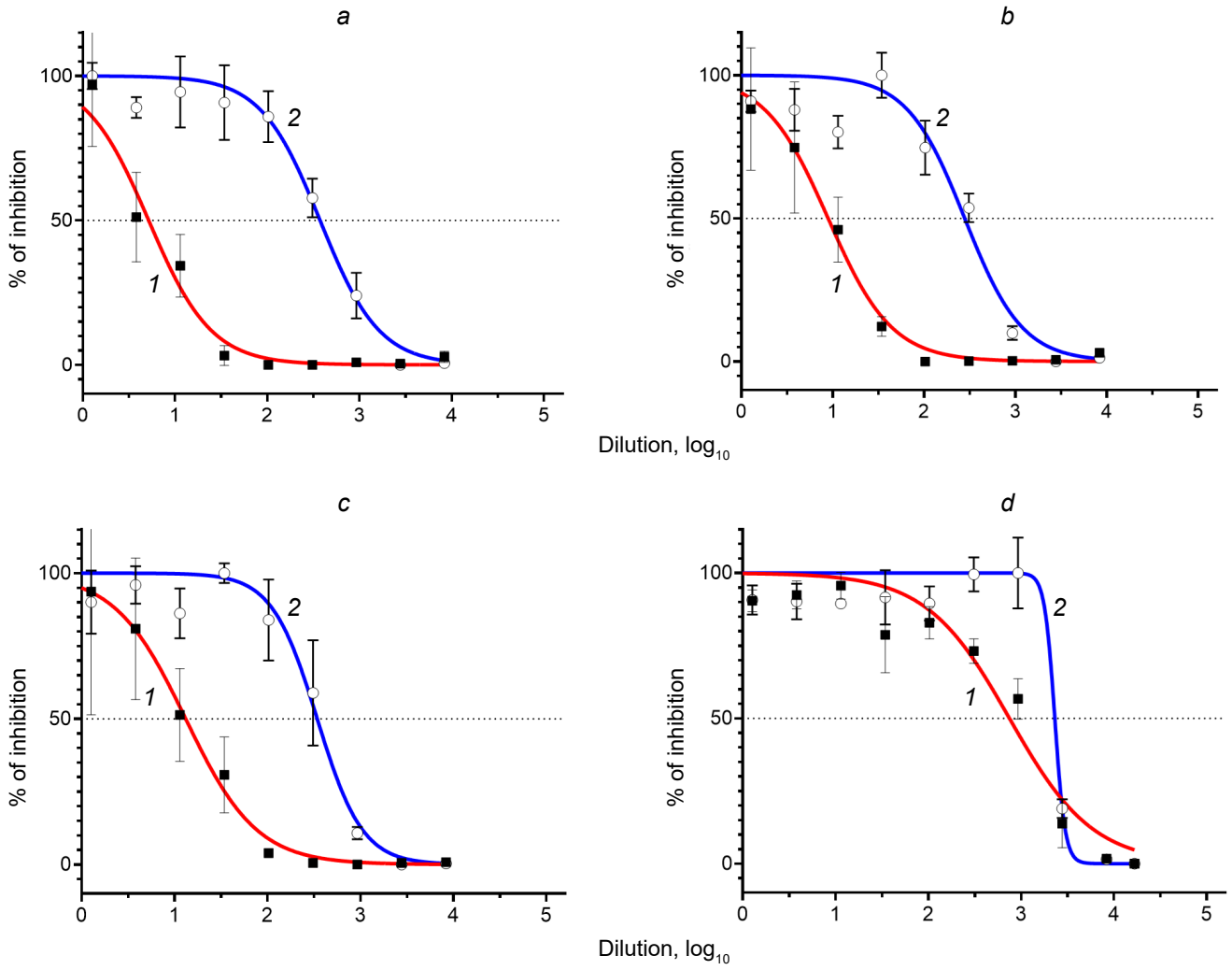

Fig. 3. Neutralizing activity of the FM1 drug (*1*) and the control drug palivizumab (*2*) against RSV strains A2 (*a*), seasonal RSV A (*b*), RSV B 9320 (*c*) and seasonal RSV B (*d*).

The titer of neutralizing antibodies was determined in 3 independent repeats, for each point the graph shows the mean value of the normalized percentage of inhibition ± standard deviation.

Comparative analysis of the neutralizing activity of the drug FM1 and the control drug palivizumab against A and B RSV subtypes

RSV strain	Mean of IC_{50}, ng/mL (95% confidence interval)	
	FM1	palivizumab
RSV A2	5,186* (3,858–6,986)	374,2 (256,1–538,5)
RSV A seasonal	8,896* (6,196–12,55)	278,4 (190,7–395,9)
RSV B 9320	13,18* (8,491–20,42)	342,7 (250,3–460,3)
RSV B seasonal	748,2* (530,1–1030)	2306 (—)

Note. The distribution of the obtained IC values did not differ significantly from normal (Shapiro–Wilk test, $p > 0.05$).
*$p < 0.01$ between group means compared with palivizumab, Student's t-test.

L1 and L2 sequences, we were guided by the fact that in the case of L1, the consensus Kozak sequence, which plays an important role in translation amplification in eukaryotes, was retained in the insert [32].

A 2-plasmid expression system based on the pVAX1 vector was selected for high-throughput expression and production of FM1 rMCA in eukaryotic cell lines. Genetically engineered constructs for the heavy and light chains of rMCA FM1 containing the variable and constant regions of the antibody, as well as signal peptides, were obtained, the cotranslation of which generates full-length rMCA FM1.

FM1 rMCA was accumulated and produced by transient expression. The eukaryotic cell line CHO-K1 was chosen as a producer of FM1 antibody. By transfection of these cells with a 2-plasmid vector system, a transient CHO-producer of rMCA FM1 was obtained. The transfected cells were adapted to selective conditions to obtain more antibody-producing clones. Long-term culturing of the temporary producer was performed to accumulate FM1 rMCAs, which were then purified from the culture fluid by affinity chromatography using modified protein A as a ligand. The absence of visible impurities was confirmed by protein electrophoresis in PAGE.

The main method for characterizing the specific activity of MCAs is the evaluation of their neutralizing activity against infectious virus by biological neutralization. Microneutralization reactions are a group of techniques that are based on the counting of registered indicators: inhibition of the development of the cytopathic action of the virus by ELISA [33], suppression of plaque formation [34, 35], spectrophotometric determination of cell viability [36], or signal reduction when a fluorescent/fluorescent reporter virus is used as an antigen [37].

Since the result obtained by the biological neutralization method is influenced by many variables, such as the type of cell lines used, method of detection, duration of incubation, etc., to study the specific activity of an MCA preparation it is necessary to use a comparison preparation with known characteristics, which makes it possible to determine the relative activity of the tested preparation. In our study we proved the biological activity of rMCA FM1 in comparison with the registered analog drug. A commercial drug based on humanized MCA Synagis (palivizumab) was used as an external positive control. The specific activity of rMCA FM1 against purified virus was confirmed by ELISA by sorption of formalin-inactivated RSV preparation onto a substrate, as well as by in-cell ELISA by infection of Vero cells with RSV A2 and RSV B 9320 strains at different doses.

The specific activity of FM1 antibody against infectious virus was demonstrated in microneutralization reaction with RSV of different subtypes. Dose-dependence curves were constructed and 50% inhibitory concentration was determined. The rMCA FM1 preparation was shown to have increased viral neutralizing activity compared to the control preparation palivizumab against RSV subtypes A and B — both reference and seasonal strains. Thus, the IC_{50} values of the tested FM1 antibody sample were significantly lower compared to the external positive control against all tested strains: for RSV A2 — approximately 72-fold, seasonal RSV A — 31-fold, RSV B 9320 — 26-fold, seasonal RSV B — 3-fold.

The average inhibitory concentration of the comparison drug palivizumab in the presented study with respect to the reference strain RSV A2 amounted to 0.374 μg/mL, which is in agreement with the previously published values and is an additional factor of validity of the obtained results. Thus, the IC_{50} value against the reference strain of RSV Long in different studies ranged from 0.353 [38] to 0.453 μg/mL [39], and the specific activity (the concentration required to reduce the size of plaques by 60%) of palivizumab against RSV A2 was 0.57 μg/mL [40].

Thus, we have obtained a candidate anti-RSV drug based on human rMCAs, which is capable of specific binding of purified RSV of both serotypes circulating in the human population, and has increased viral neutralizing activity against both reference and seasonal strains of RSV subtypes A and B, compared to the control drug palivizumab.

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Conclusion

In this study, rMCA FM1 to RSV glycoprotein F was developed and obtained, which has increased virus-neutralizing activity against reference and seasonal strains of RSV subtypes A and B compared to palivizumab. Currently, the team of authors is working on obtaining a stable rMCA FM1 clone with high productivity and viability, as well as studying the therapeutic efficacy of rMCA FM1 in a sublethal RSVI model in mice.

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Characteristics of the monkeypox virus isolate obtained from the first patient in Russia and its sensitivity to 7-[N-(4-trifluoromethylbenzoyl)-hydrazinocarbonyl]-tricyclo-[3.2.2.0^2,4]non-8-en-6-carboxylic acid

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Abstract

Introduction. Since early May 2022, more than 90,000 cases of monkeypox virus infection have been reported in more than 70 countries around the World. This is the largest outbreak of monkeypox ever recorded outside of Africa.

The **aim** of the study is to confirm the first case of monkeypox in Russia, to isolate and sequence a new strain of monkeypox virus (MPXV), and to assess its sensitivity to the 7-[N-(4-trifluoromethylbenzoyl)-hydrazinocarbonyl]-tricyclo-[3.2.2.0^2,4]non-8-en-6-carboxylic acid (NIOCH-14) antipox drug.

Materials and methods. The biological materials obtained from the affected area of the skin (contents of vesicles), a nasopharyngeal smear, sputum and venous blood from a patient with suspected monkeypox were used. The disease was confirmed by PCR followed by determination of the nucleotide sequence of viral DNA by sequencing. Isolation of the new MPXV strain from clinical samples was carried out in Vero E6 cells. The antiviral effectiveness of NIOCH-14 against the new MPXV strain was assessed using an adapted spectrophotometric method.

Results. A diagnostic study of the biological samples of a patient who returned from a tourist trip to European countries with complaints of skin rashes all over the body revealed MPXV DNA. A new strain of MPXV was isolated from vesicles in Vero E6 cells, and the genomic sequence MPXV-pustule S45 was assembled using high-throughput parallel sequencing (NGS).

Discussion. The effectiveness of the finished dosage form of NIOCH-14 against the new strain of MPXV based on the results of determining the 50% virus inhibitory concentration (IC_{50}) was 0.02 μg/mL, and the selectivity index (SI) was > 15,000.

Conclusion. In this study, the pathogen of monkeypox was detected and identified using real-time PCR, NGS and electron microscopy, and the first imported case of this disease in Russia was confirmed. It has been proven that the drug NIOCH-14 exhibits high antiviral activity *in vitro* against the new MPXV strain.

Keywords: *monkeypox virus, genomic sequencing of viruses, PCR, cytopathic effect, antiviral activity, NIOCH-14*

Ethics approval. The study was conducted with the informed consent of the patient. The research protocol was approved by the Ethics Committee of the State Scientific Center of Virology and Biotechnology "Vector" (protocol No. 5a, July 21, 2022).

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Оригинальное исследование
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Характеристики изолята вируса оспы обезьян, полученного от первого заболевшего в России, и его чувствительность к 7-[N-(4-трифторметилбензоил)-гидразинокарбонил]-трицикло-[3.2.2.0^2,4]нон-8-ен-6-карбоновой кислоте

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Аннотация

Введение. С начала мая 2022 г. было зарегистрировано более 90 тыс. случаев заражения вирусом оспы обезьян (ВОО) в более чем 70 странах мира. Это самая крупная из зарегистрированных вспышек оспы обезьян, вышедшая за пределы Африки.

Цель работы — подтверждение первого случая оспы обезьян в России, выделение и секвенирование изолята ВОО, а также оценка его чувствительности к противооспенному препарату — 7-[N-(4-трифторметилбензоил)-гидразинокарбонил]-трицикло-[3.2.2.0^2,4]нон-8-ен-6-карбоновой кислоте (НИОХ-14).

Материалы и методы. В работе использовали биологические материалы, полученные из поражённого участка кожи (содержимое везикул), мазка из носоглотки, мокроты и венозной крови пациента с подозрением на оспу обезьян. Заболевание подтверждали методом полимеразной цепной реакции (ПЦР) с последующим определением нуклеотидной последовательности вирусной ДНК методом секвенирования. Штамм ВОО из клинических образцов выделяли в культуре клеток Vero E6. Противовирусную эффективность НИОХ-14 в отношении изолята ВОО оценивали с использованием адаптированного спектрофотометрического метода.

Результаты. Диагностическое исследование биологических образов пациента, вернувшегося из туристической поездки по европейским странам, с жалобами на кожную сыпь по всему телу выявило в них ДНК ВОО. Изолят ВОО был выделен из содержимого везикулы в культуре клеток, генетическая последовательность MPXV-pustule S45 была собрана по результатам проведения высокопроизводительного параллельного секвенирования.

Обсуждение. Эффективность противовирусного действия готовой лекарственной формы НИОХ-14 в отношении нового штамма ВОО по результатам определения 50% вирусингибирующей концентрации составила 0,02 мкг/мл, индекс селективности — > 15 000.

Заключение. В настоящем исследовании методами ПЦР в режиме реального времени, секвенирования и электронной микроскопии был выявлен и идентифицирован возбудитель оспы обезьян, из клинического образца (содержимое везикул) на культуре клеток Vero E6 был выделен изолят ВОО и, таким образом, подтверждён первый завозной случай оспы обезьян в России. Было доказано, что препарат НИОХ-14 проявляет высокую противовирусную активность *in vitro* в отношении выделенного изолята ВОО.

Ключевые слова: *вирус оспы обезьян, геномное секвенирование вирусов, полимеразная цепная реакция, цитопатический эффект, противовирусная активность, НИОХ-14*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациента. Протокол исследования одобрен Этическим комитетом ГНЦ ВБ «Вектор» (протокол № 5а от 21.07.2022).

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Introduction

More than 60 years have passed since the discovery of the causative agent of a particularly dangerous zooanthroponous viral infection in humans, the monkeypox virus (MPXV), which belongs to the same *Orthopoxvirus* genus (*Poxviridae* family) as the smallpox virus and is highly lethal in humans (up to 17%) [1–3]. MPXV genomic sequencing has shown the existence of two branches of this virus: The West African clade and the Central African (Congo Basin) clade, each of which causes the MPXV disease, but the West African clade virus is considered less dangerous [4, 5]. Since its discovery, the disease has been endemic only to Central and West Africa [6–8]. However, since the beginning of May 2022, monkeypox has spread far beyond the African continent, prompting the World Health Organization to declare the 2022 outbreak a public health emergency of international concern[1]. Monkeypox is now spreading globally, with more than 90,000 infections already reported in more than 70 countries around the world. This is the largest outbreak of monkeypox ever seen in Africa and beyond.

Smallpox drugs play an important role in the control of monkeypox. A new specific drug for the treatment of monkeypox, Tecovirimat [9], an inhibitor of the viral protein VP37, has been approved in Europe, the United States, Canada, and several other countries. This drug is currently widely used for the therapy of this disease [10], but data on the identification of resistant MPXV variants to this drug have emerged [11–13]. The drug 7-[N-[N-(4-trifluoromethylbenzoyl)-hydrazinocarbonyl]-tricyclo-[3.2.2.0^2,4] non-8-en-6-carboxylic acid (NIOCH-14), which has a similar mechanism of action as Tecovirimat, has been registered in Russia [14].

Human monkeypox is no longer a rare disease and represents a public health problem, so it is important to have access to registered drugs that can effectively counteract this disease.

The aim of this study was to confirm the first reported case of MPXV in Russia, to isolate and sequencing an isolate of MPXV, and to assess its sensitivity to the domestic anti-monkeypox drug NIOCH-14.

Materials and methods

Cell culture

The Vero E6 cell culture obtained from the collection of cell cultures of the State Scientific Center of Virology and Biotechnology "Vector" was used. DMEM medium in the presence of 2% fetal bovine serum supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL) was used as a maintenance medium for MPXV cultivation.

Patient, test samples

The patient was a male, 28 years old, with clinical signs of monkeypox, from whom material from the affected skin (contents of vesicles), nasopharyngeal swab and sputum, and venous blood was collected for the work. The study was conducted with voluntary informed consent of the patient. The study protocol was approved by the Ethical Committee of the State Scientific Center of Virology and Biotechnology "Vector" (protocol No. 5a dated 21.07.2022).

The work with MPXV was carried out at the Laboratory of Biosafety Level 4 of the State Scientific Center of Virology and Biotechnology "Vector".

Isolation, titration and culture of monkeypox virus

The sample with vesicle contents was diluted in 0.5 mL of DMEM medium supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/mL) before addition to the Vero E6 cell culture monolayer. Venous blood, nasopharyngeal swab and sputum samples were used without additional dilution with medium before application to the cell monolayer. Supernatant of samples obtained after centrifugation at 700g for 10 min was added 50 μL to the wells of a 96-well plate with Vero E6 cell culture monolayer. The plates were incubated at 37ºC under 5% CO_2 conditions and the appearance of cytopathic effect was monitored daily. Culture medium from the wells with the disrupted monolayer (passage 1) was collected and transferred to T-25 vials with the pre-grown Vero E6 cell culture monolayer for further incubation at 37ºC. When 80% cytopathic effect was reached in the cell monolayer, the vials were frozen/thawed 3 times and the resulting virus-containing suspension was clarified by centrifugation at 1200g for 10 min (passage 2). The virus-containing suspension of passage 2 was titrated by the plaque method in 24-well plates with *Vero E6* cell culture monolayer and used for viral DNA extraction and subsequent analysis using high-throughput sequencing.

Obtaining whole genome nucleotide sequences

MPXV DNA was isolated from source material by phenol-chloroform method[2].

The concentration of viral DNA was measured using the Qubit dsDNA HS Assay Kit (Qubit 3.0, Thermo Fisher Scientific), then the solution was used to prepare libraries for high-throughput sequencing on the Illumina platform. The Y-adapter ligation method (Illumina) was used to prepare libraries. Sequencing was performed on

[1] Multi-country monkeypox outbreak: situation update. World Health Organization, June 17, 2022. URL: https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON393
Multi-country monkeypox outbreak: situation update. World Health Organization, June 27, 2022. URL: https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON396

[2] Organization of laboratory operations using nucleic acid amplification methods when working with materials containing microorganisms of pathogenicity groups I–IV: methodological guidelines. Moscow; 2010. 51 p.

a MiSeq sequencer (Illumina) using the MiSeq Reagent Kit v2 (500-cycles) sequencing kit (Illumina).

Testing samples for the presence of MPXV DNA

Confirmatory diagnostic testing was performed using a reagent kit for detection of genetic markers (DNA) of orthopoxviruses, including MPXV, by real-time polymerase chain reaction (qPCR) using the Vector-MPCR rv-Ospa reagent kit for detection of DNA of smallpox, monkeypox, cowpox, and vaccinia viruses with hybridization-fluorescence detection in real time (Vector-MPCR rv-Ospa) (Vector, RZN No. 2016/3685).

Tested drug, inhibitor of orthopoxvirus replication

The domestic drug against MPXV NIOCH-14, series 010919, was used in the study [14].

Method for determination of antiviral activity of the preparation NIOCH-14 in vitro

Cytotoxicity and antiviral efficacy of NIOCH-14 (series 010919) were evaluated using the colorimetric method [15]. The wells of 96-well plates containing a monolayer of Vero E6 cells (~40 thousand cells per well) were firstly filled with 100 μL of serial dilutions of solutions prepared from the finished dosage form of NIOCH-14. Then 100 μL of MPXV dilution was added at a dose of 800 BOU/well (multiplicity of infection ~ 0.02 viral particles per cell), causing 100% cell destruction in the control monolayer without the drug, which occurs when cells are incubated with the virus 6 days after infection[3]. The cytotoxic activity of the drug was determined by its effect on cell destruction in the wells of the plate in which the virus was not introduced. Monolayers of cells in the wells of the plate, in which the virus was introduced without the drug (virus control) and monolayers of cells in the wells in which neither the virus nor the drug was introduced (cell control) were used as controls.

The capsule of the finished dosage form of the preparation NIOCH-14 was opened, the contents were poured into a vial. 10 mL of dimethyl sulfoxide was added to the contents of the capsule. The resulting solution was mixed in equal volumes with the nutrient medium with antibiotic. To evaluate cytotoxicity against Vero E6 cell culture and antiviral activity against MPXV, serial 3-fold dilutions of the drug were prepared, 8 dilutions were used starting from a concentration of 600 μg/mL. To evaluate the antiviral activity of the drug, 8 dilutions starting from 2 μg/mL were used.

NIOCH-14 dilutions were added in the volume of 100 μL into the wells of 96-well plates with cell culture. After incubation at 37ºC for 2 h (prophylactic scheme), 100 μL of culture medium was added to the wells to assess cytotoxicity, and 100 μL of MPXV dilution was added to assess antiviral activity. As a result, the total volume of liquid in each well was 200 μL, the initial concentration of the drug in the wells for cytotoxicity was 300 μg/mL, and for antiviral activity was 1.00 μg/mL.

After 6 days of incubation at 37ºC, the cell monolayer was stained with neutral red dye for 2 hours. After removing the dye and washing the wells from its unbound fraction, 0.1 mL of lysing buffer was added to release the dye from the absorbed cells. The optical density of the resulting solution, which depends on the number of cells in the monolayer that were not destroyed by the drug or virus, was measured using an Emax spectrophotometer (Molecular Devices) at a wavelength of 490 nm. Using the SoftMax 4.0 program (Molecular Devices) we calculated the 50% toxic concentration (TC_{50}, μg/mL) of the drug, at which 50% of cells in the uninfected monolayer are destroyed, and 50% inhibitory concentration (IC_{50}, μg/mL) of the drug, at which 50% of cells in the infected monolayer are not destroyed (remain viable). Based on TC_{50} and IC_{50}, the selectivity index (SI) of the drug was determined: $SI = TC_{50}/IC_{50}$.

Electron microscopic examination

MPXV-infected Vero E6 cells were separated using a rubber spatula and fixed in an equal volume of 8% paraformaldehyde solution for 1 day. After centrifugation (1500 rpm, 10 min) and washing three times, the precipitate was further fixed with 1% OsO_4 solution. Dehydration, impregnation and casting in epon-araldite mixture were performed according to the generally accepted method. Ultrathin sections were prepared on a microtome (Reichert-Jung), stained with uranyl acetate and lead citrate. Sections were examined in a JEM 1400 electron microscope (Jeol). Photography and image analysis were performed using a Veleta digital camera and iTEM software package (SIS).

Statistical and bioinformatics analysis of data

MPXV titer data were presented as mean value and its standard deviation ($n = 4$).

Bioinformatics analysis of virus fragment sequences was performed using MIRA v. 4.9.6, BWA v. 0.7.15, IGV v. 4.9.6, BWA v. 0.7.15, IGV v. 2.3.78, Samtools v. 1.3.1, Bcftools v. 1.62, SnpEff v. 5.2. Whole-genome sequence alignment was performed using the MAFFT algorithm v. 7.505. The MPXV genome MPXV-M5312_HM12_Rivers (NC_063383.1), belonging to the West African clade, was used as a reference nucleotide sequence. A phylogenetic tree was constructed using the neighbor-joining method (IQ-Treev.2.1.4, GTR+G+I nucleotide substitution model) using *Orthopoxvirus* reference (RefSeq) sequences ($n = 9$). In addition, all MPXV sequences available

[3] Sergeev A.A., Kabanov A.S., Bulychev L.E., et al. Method for evaluating the activity of therapeutic and prophylactic agents against the smallpox virus. Patent 2522483 RF (A61K 35/76, A61P 31/12, C12N 7/00).

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by July 2024 from samples collected between January 2018 and August 2022 were downloaded from the GISAID database. The total number of sequences used for analysis was 2,289. Genome regions with missing positions in the sequence analyzed were excluded from the analysis.

Results

Detection of MPXV in clinical samples

The study was conducted with clinical specimens obtained from a citizen of the Russian Federation who returned from a tourist trip to European countries — Spain, Portugal, etc. (where at that time there was an increase in the incidence of monkeypox. A few days after his return from abroad (08.07.2022), the patient went to a medical institution with complaints of a rash all over his body. Based on clinical symptoms and epidemiologic analysis, the patient was suspected to have monkeypox. Nasopharyngeal swab and sputum, as well as the contents of vesicles collected from the patient upon his admission to the medical institution and transferred to the laboratory of the State Scientific Center of Virology and Biotechnology "Vector" for PCR examination were positive for MPXV DNA content, which confirmed the diagnosis of monkeypox. At the same time, MPXV DNA was not detected in the patient's venous blood sample also submitted for testing (**Table**).

MPXV DNA from patient samples was subjected to high-throughput sequencing and subsequent bioinformatics analysis. The genomic sequence MPXV-pustule S45 (sequence number vect2SM413009in the VGARus database) was assembled based on the reads obtained from sequencing of MPXV DNA isolated from the patient's clinical material. The length of the obtained sequence was 197,203 bp (98.77%), the average depth of coverage was 11.72, the number of undecoded nucleotides was 3,346, and the total number of reads per target genome was 27,168.

Phylogenetic analysis of the MPXV-pustule_S45 sequence (**Fig. 1**) showed that the studied MPXV isolate belongs to genetic clade IIb, lineage B.1. The MPXV sequences isolated in 2022 from patients from the USA, Peru, and Western European countries (Germany, Portugal and Ireland) are the most genetically similar to the investigated sample.

Analysis of the nucleotide sequences of MPXV-pustule_S45 in comparison with the MPXV reference sequence NC_063383.1 showed the presence of 66 nucleotide substitutions, 32 of which are missense mutations, and one substitution leads to a frameshift (gene OPG055). Furthermore, the MPXV-pustule_S45 sequence examined contains an E353K mutation in the F13L protein (OPG057 gene, genomic position 39139). However, no mutations causing resistance to Tecovirimat were detected when analyzing MPXV-pustule_S45.

Isolation of monkeypox virus in cell culture

To isolate viable MPXV from clinical samples, samples with the contents of vesicles from a diseased person were used. Two days after inoculation of Vero E6 cell culture, a slight change in cell morphology was observed in comparison with uninfected control cell culture, which became manifest as cytopathic action on the 5th day after infection. Two consecutive passages were carried out to develop a working virus stock and deposit it in the State Collection of Viral Infectious Diseases and Rickettsioses Pathogens, functioning at the State Scientific Center of Virology and Biotechnology "Vector", as St. Petersburg-22 MPXV strain. The efflux titer of the virus in the culture fluid was 5.9 ± 0.3 log10 BOU/mL.

Electron microscopic examination

A sample of Vero E6 cells infected with the St. Petersburg-22 MPXV strain was examined using electron microscopy (**Fig. 2**).

Ultrastructural analysis of infected cells showed the presence of orthopoxvirus-specific viral factories, which after 48 h occupied most of the cytoplasm of infected Vero E6 cells. Viral particles, typical for orthopoxviruses, 48 h after infection of Vero E6 cells, were present at all stages of their formation cycle, with mainly immature virions predominating. At the same time, 72 h after infection of Vero E6 cells, mature viral particles were predominantly observed.

Antiviral activity of NIOCH-14 in in vitro experiments

The St. Petersburg-22 strain of MPXV was used to evaluate the antiviral efficacy of NIOCH-14. The results are presented in **Fig. 3**.

The TC_{50} for NIOCH-14 was > 300 μg/mL (> 731 μM) and the IC_{50} of NIOCH-14 for the St. Pe-

Presence of MPXV DNA in samples tested with the Vector-MPCR rv-Ospa reagent kit

Sample type	Indicator	Study results
Vesicle contents	MPXV DNA	Found, Ct = 19.72
Nasopharyngeal swab and sputum	MPXV DNA	Found, Ct = 16.18
Venous blood	MPXV DNA	Not found, Ct > 40*

Note. *According to the manufacturer's instructions, a Ct value > 40 is considered a negative test result.

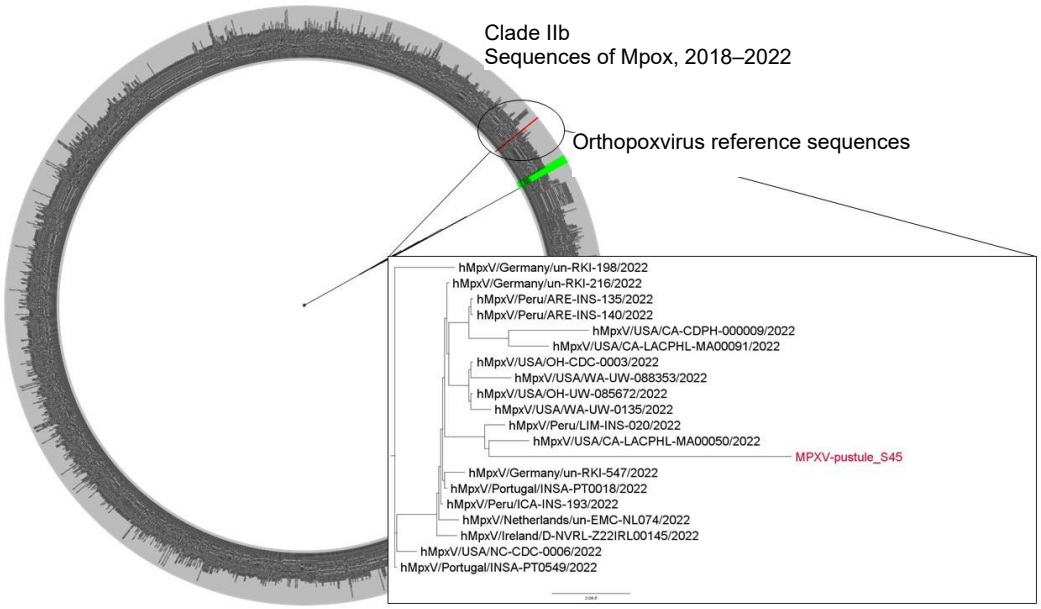

Fig. 1. Phylogenetic tree constructed using the maximum likelihood method.
Mpox sequences loaded from GISAID are highlighted on the circular cladogram. The position of the investigated sequence MPXV-pustule_S45 and its closest sequences is circled. A detailed cladogram is given.

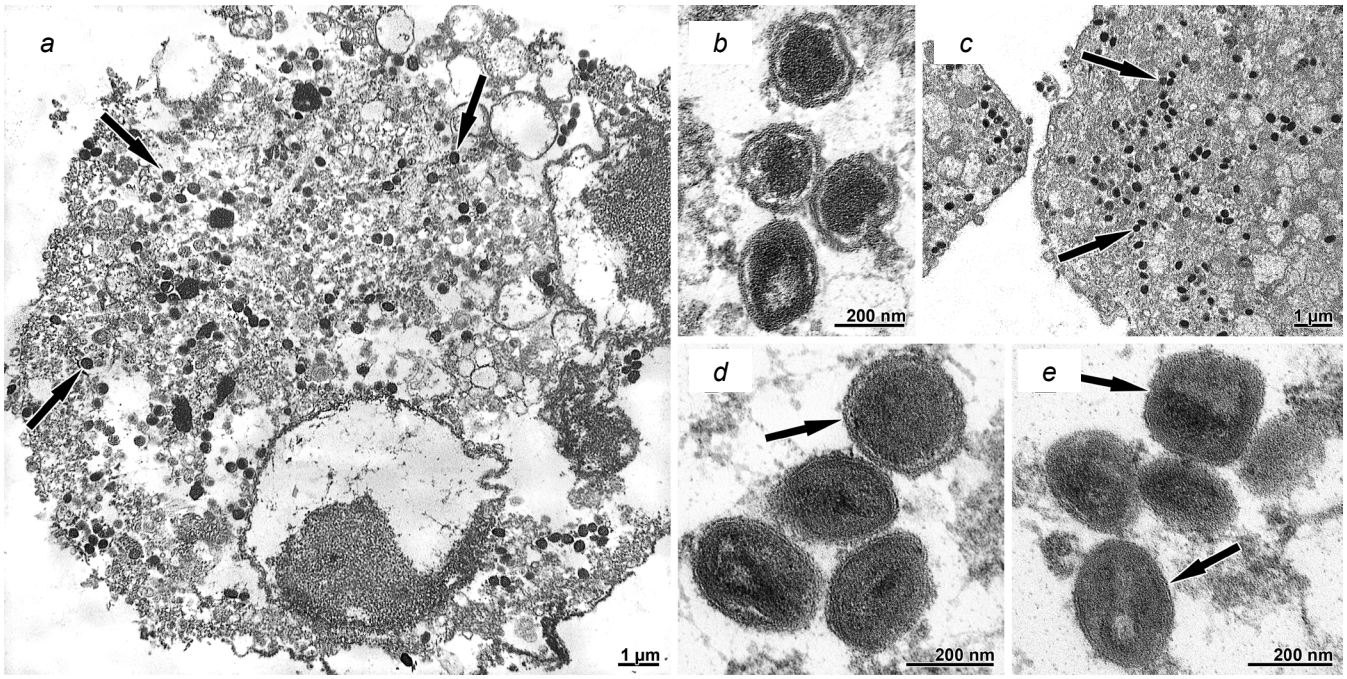

Fig. 2. Vero E6 cells infected with a new St. Petersburg-22 MPXV strain.
a, *b* — 48 h after infection, the viral factory occupies most of the cell cytoplasm; viral particles are represented at all stages of the formation cycle (arrows); immature virions are prevalent (image *b*).
Images *c*–*e* — 72 h after infection, image *c* shows the prevalence of mature viral particles (arrows). In image *d*, the arrow indicates an immature viral particle. Image *e* shows the typical morphology of a mature orthopoxvirus virion.

tersburg-22 strain of MPXV was 0.016 ± 0.009 µg/mL (0.039 ± 0.009 µM), whereas the SI was > 18,750.

Discussion

The monkeypox outbreak, which has been declared a public health emergency of international concern by WHO, has been spreading globally since early May 2022, affecting predominantly men who have sex with men. Cases have been reported in more than 70 countries worldwide, posing a public health threat of international concern. According to WHO recommendations, laboratories use samples from skin lesions,

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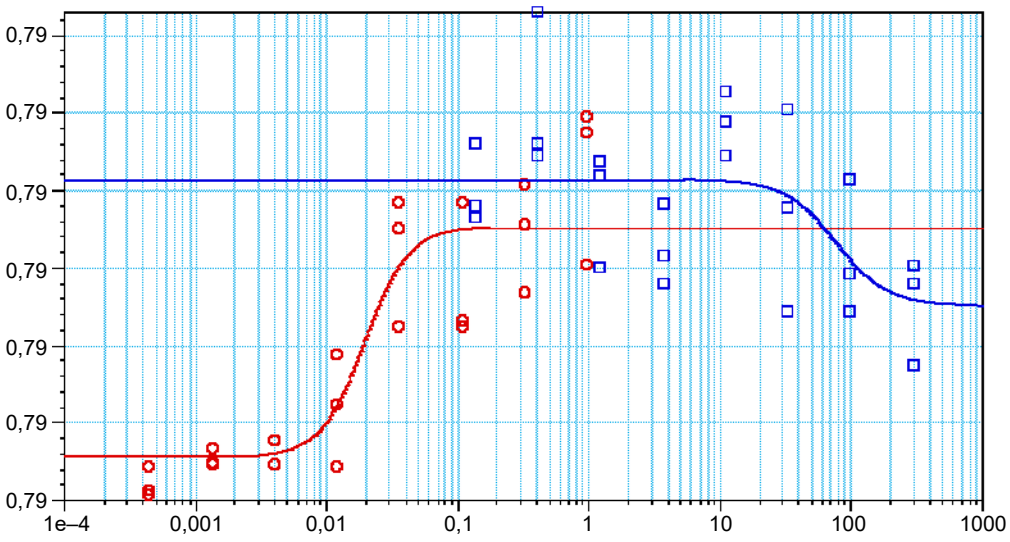

Model	Parameters of model				
y = ((A − D)/(1 + (x/C)^B)) + D	A	B	C	D	R^2
activity	0,244	2,596	0,02	0,542	0,825
toxicity	0,604	2,201	78,476	0,44	0,287

Fig. 3. Diagram of cytotoxicity (TC_{50} > 300 μg/mL) and antiviral activity of the finished dosage form of NIOCH-14 against the St.Petersburg-22 MPXV strain (IC_{50} = 0.02 μg/mL) in Vero E6 cell culture (SoftMax 4.0 program).
On the X-axis — drug concentration (μg/mL) in logarithmic scale of measurement; on the Y-axis — optical density (optical density units) in linear scale of measurement.

pharyngeal and nasopharyngeal swabs and blood for diagnostic tests[4]. In this case, the virus is detected most often in samples from skin lesions and less often in blood, which is probably due to the fact that viremia occurs during a very early and short period of infection and usually blood contains less virus than skin lesions [16]. Examination of samples (nasopharyngeal swabs, sputum, and vesicle contents) obtained from the first monkeypox patient registered in Russia also allowed us to detect MPXV DNA in nasopharyngeal swabs, sputum and vesicle contents, and to isolate a viable virus culture only from vesicle contents, while no virus was detected in the blood sample.

Sequencing data analysis of MPXV-pustule S45 sequence of the St. Petersburg-22 MPXV strain showed that it belongs to genetic clade IIb, lineage B.1. The genome of the St. Petersburg-22 strain is closely related to 19 MPXV isolates detected in summer and fall of 2022 in different countries.

According to studies, it is known that a person usually becomes infectious after the onset of disease symptoms [17]. However, recent studies on the spread of MPXV have established cases of virus excretion by patients without symptoms of the disease [18]. Thus, the infection of the first monkeypox patient in Russia, given that the incubation period of the disease can be up to 3 weeks, could have occurred through contact with both an asymptomatic patient and a previously ill patient, since the virus can be excreted with seminal fluid, oropharyngeal and anogenital secretions of the patient for some time after the disappearance of symptoms [19]. This is supported by the fact that contact with a monkeypox patient with a pronounced clinical picture of the disease was hardly possible due to the usually pronounced pain syndrome.

The first symptoms of monkeypox in a patient from Russia were diagnosed on 06.07.2022. Given the incubation period, which can range from 3 days to 3 weeks [20], his probable infection occurred during the period from mid-June to early July 2022. According to GISAID data, among the phylogenetically close sequences identified during this time period, the epidemiologically related to the MPXV strain St. Petersburg-22 patients could be those with the sequences hMpxV/USA/CA-CDPH-000009/2022 (identified on 06/30/2024), hMpxV/Ireland/D-NVRL-Z22IRL00145/2022 (identified on 06/17/2024) if, for example, patients were asymptomatic, or even hMpxV/Portugal/INSA-PT0018/2022 (identified on 06/01/2024), if infection occurred from a recently recovered individual. However, as previously indicated, infection most likely occurred through contact with an asymptomatic Mpox carrier. It is also interesting to note that the genetically closest sequences hMpxV/Peru/LIM-INS-020/2022 and hMpxV/USA/CA-LACPHL-MA00050/2022 were identified around the same time as the MPXV sequence

[4] World Health Organization. Laboratory testing for the monkeypox virus. Interim guidance. URL: https://apps.who.int/iris/rest/bitstreams/1425052/retrieve (data of access: 23.05.2022).

from the first patient in Russia, namely July 12 and 20, 2022, respectively. It is possible that the patients from whom viruses with these sequences were obtained are linked to the Russian patient by a common source of infection.

To isolate a live culture of MPXV, Vero cell culture is traditionally used, which is highly sensitive to orthopoxviruses. The virus titers reach the level of 5 log10 $TCID_{50}$/mL when cultured for 6 days [21]. MPXV isolated from biological samples obtained from the first Russian patient with monkeypox, when cultured in Vero cell culture, reached a titer of 5.9 log10 CFU/mL in the culture fluid already 5 days after infection of the cell monolayer. At the same time, ultrastructural analysis of the infected monolayer of Vero E6 cell culture demonstrated the presence of characteristic signs of virus multiplication in cells and the presence of viral particles of classical type, characteristic of MPXV, similar to the ultrastructural picture in infected cells detected by other researchers [21].

The widespread use of Tecovirimat for the treatment of monkeypox patients led to the emergence of information about the discovery of MPXV variants with drug resistance to this drug [13, 22]. Mutations in the F13L gene, homologous in orthopoxviruses, have been found to reduce the sensitivity of the virus to Tecovirimat [11]. The study of the genetic structure of the Mpox strain isolated by us did not establish the presence of known resistance mutations to Tecovirimat, and the evaluation of the strain's sensitivity to the domestic drug NIOCH-14 showed high inhibitory activity of this drug, comparable to that previously estimated by us for the reference MPXV strain (TC_{50} > 100 μg/mL, IC_{50} = 0.013 μg/mL, SI > 7700) [15]. This proves that the preparation NIOCH-14 has high antiviral activity *in vitro* against the St. Petersburg-22 MPXV strain detected in the first patient in Russia.

Conclusion

The approaches used in this study made it possible to confirm the first imported case of monkeypox in Russia, to isolate virus from biological samples, to characterize the culture and biological properties of the St. Petersburg-22 MPXV strain, and to deposit the strain in the State Collection of Viral Infectious Pathogens and Rickettsioses of the State Scientific Center of Virology and Biotechnology "Vector". Furthermore, studies of the antipox activity of the domestic drug NIOCH-14 showed that it exhibits high antiviral efficacy *in vitro* against the first MPXV strain identified in Russia and can be used for the treatment of monkeypox patients.

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Pathogenic potential of ornithogenic *Escherichia coli* strains detected in the Earth's polar regions

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Abstract

Introduction. Pathogenic strains of *Escherichia coli* are an important object of surveillance within the One Health concept in the wild, agriculture and human society.

Migratory bird colonies and high latitude avian colonies may be points of active intraspecies and interspecies contact between different animal species, accompanied by the spread of pathogens. At the same time, the phylogeography of *E. coli* in relation to the presence of natural foci of colibacillosis in polar regions remains virtually unstudied.

The aim of this study was to assess the pathogenic potential of *E. coli* strains from the polar regions of the Earth, based on the analysis of the genomes of these bacteria from typical ornithogenic ecosystems of the Arctic and Antarctic.

Materials and methods. The study used collections of *E. coli* isolated from ornithogenic biological material during expeditions to high latitude areas of the Arctic (archipelagos of Novaya Zemlya, Franz Josef Land, Svalbard) and Antarctic (Haswell Archipelago). 16 cultures associated with avian *E. coli* (12 polar and 4 temperate strains) were selected for genome-wide sequencing using BGI technology. The annotation of the genomes focused on the identification of genes for pathogenicity factors and antimicrobial resistance, as well as the identification of strains belonging to individual genetic lineages using the cgMLST method.

Results. The annotation of the genomes allowed their assignment to different sequence types in the multilocus sequencing typing and genome-wide sequencing typing schemes. The analysis of the geographical distribution of the sequence types of polar *E. coli* strains determined by the cgMLST method showed their global representation in geographically distant regions of the planet. For example, cgST 133718 was observed in Antarctica (strain 17_1myr) and in the UK, and sequence 11903, to which strain 32-1 from the northernmost point of Novaya Zemlya belonged, was previously identified in the USA.

All strains studied were characterized by the presence of extensive virulence. Among the pathogenicity factors identified were haemolysins A, E, F, siderophores, including the yersiniabactin gene cluster, a number of adhesion, colonization and invasion factors, as well as the thermostable enterotoxin EAST-1 and genes that characterize enteroaggregative strains of *E. coli* (the virulence regulator gene *eilA* and enteroaggregative protein (air)). One of the Arctic strains (33-1) had determinants of antibiotic resistance, in particular the extended-spectrum beta-lactamase gene TEM-1b and the Tn1721 transposon, including tetracycline resistance genes (tetA-TetR), were detected in its genome.

Conclusion. The results of the study indicate the circulation of *E. coli* strains with strong pathogenic potential in high-latitude Arctic and Antarctic ornithogenic ecosystems. The analysis of genomic data indicates the presence of geographically widespread genetic lineages in these regions, which justifies the importance of monitoring epidemic clones of *E. coli*, along with monitoring for other pathogens, in bird colonies in high-latitude areas.

Keywords: *Arctic, Antarctica, Escherichia coli, genome sequencing, pathogenicity factors, ornithogenic ecosystems*

Ethics approval. The procedure of biological material sampling was carried out in accordance with generally accepted ethical normsю The research protocol was approved by the Ethics Committee of the which is confirmed by the decision of the Local Ethics Committee of North-Western State Medical University named after I.I. Mechnikov (Protocol No. 3, March 13, 2024).

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Оригинальное исследование
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Патогенный потенциал орнитогенных штаммов *Escherichia coli*, выявленных в полярных регионах Земли

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Аннотация

Введение. Патогенные штаммы *Escherichia coli* являются важным объектом мониторинга в природе, сельском хозяйстве и человеческом обществе в рамках концепции «Единого здоровья». Колонии мигрирующих птиц и птичьи базары в высоких широтах могут быть точками активных внутривидовых и межвидовых контактов между различными видами животных, сопровождающихся распространением микроорганизмов. В то же время филогеография *E. coli* в контексте наличия природных очагов колибактериозов в полярных регионах практически не изучалась.

Цель работы: оценка патогенного потенциала штаммов *E. coli*, распространённых в полярных регионах Земли, на основе анализа геномов данных бактерий из выборки, характеризующей типичные орнитогенные экосистемы Арктики и Антарктики.

Материалы и методы. В работе были использованы штаммы *E. coli*, выделенные из орнитогенного биологического материала в ходе экспедиций на высокоширотные территории Арктики (архипелаги Новая Земля, Земля Франца-Иосифа, Шпицберген) и Антарктики (архипелаг Хасуэлл). Из них 16 штаммов, ассоциированных с птицами (12 полярных штаммов и 4 штамма, выделенных в умеренных широтах), были отобраны для полногеномного секвенирования с использованием технологии BGI. Аннотирование геномов было сфокусировано на идентификации генов, кодирующих факторы патогенности и устойчивости к антимикробным препаратам, а также на определении принадлежности штаммов к отдельным серотипам и генетическим линиям, в том числе на основе использования метода cgMLST.

Результаты. Проведённое аннотирование геномов *E. coli* позволило установить их принадлежность к различным сиквенс-типам в схемах мультилокусного секвенирования-типирования и полногеномного секвенирования-типирования. Анализ географического распространения сиквенс-типов «полярных» штаммов *E. coli*, определённых методом cgMLST, продемонстрировал их глобальную представленность. Так, например, cgST 133718 был отмечен в Антарктиде (штамм 17_1myr) и ранее — в Великобритании, а сиквенс-тип 11903, к которому принадлежал штамм 32-1 из самой северной точки Новой Земли, был ранее выявлен в США. Все изученные штаммы характеризовались наличием обширного вирулома. В числе выявленных генов факторов патогенности обнаружены гены гемолизинов A, E, F, сидерофоры, включая иерсиниабактиновый кластер генов, ряд генов факторов адгезии, колонизации и инвазии, а также ген термостабильного энтеротоксина EAST-1 и гены, маркирующие энтероаггрегативные штаммы *E. coli*: ген регулятора вирулентности *eilA* и энтероаггрегативный белок (air). Один из «арктических» штаммов (33-1) характеризовался наличием детерминант устойчивости к антибиотикам, в частности, в его геноме был детектирован

ген бета-лактамазы расширенного спектра TEM-1b и транспозон Tn1721, включающий гены устойчивости к тетрациклинам (tetA-tetR).

Заключение. Результаты исследования свидетельствуют о циркуляции в орнитогенных экосистемах высокоширотной Арктики и Антарктики штаммов *E. coli*, обладающих выраженным патогенным потенциалом. Анализ геномных данных свидетельствует о распространении в этих регионах генетических линий, широко географически представленных, что обосновывает значимость мониторинга эпидемических клонов кишечной палочки, наряду с мониторингом других патогенов, в колониях массовых видов птиц на высокоширотных территориях.

Ключевые слова: *Арктика, Антарктика, Escherichia coli, полногеномное секвенирование, факторы патогенности, орнитогенные экосистемы*

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Introduction

Escherichia coli is a unique microorganism capable of causing infections in a wide range of clinical manifestations in humans and various animals, which determines the importance of monitoring the spread of its main pathotypes in nature, agriculture and human society [1].

One of such pathotypes monitored under the One Health concept is avian pathogenic *E. coli* (avian pathogenic *E. coli* — APEC), which belongs to the group of pathogens of extraintestinal localization (extraintestinal pathogenic *E. coli* — ExPEC) [2, 3].

Although the possibility of direct zoonotic transmission of APEC from birds to humans is debatable [4], numerous studies show that APEC are genetically similar to human ExPEC (uropathogenic *E. coli* (UPEC) and *E. coli* associated with neonatal meningitis (NMEC)). There are studies supporting the commonality of pathogenicity factors in human APEC and ExPEC isolates. For example, the virulence genes *iroN*, *traT*, *iucD*, *cvi/cva*, *ibeA*, *gimB*, *tia*, *neuC*, *kpsMTII*, *tsh*, *iss*, *sitD*, *chuA*, *fyuA*, *irp2*, *vat*, *malX* and *pic* are present in the genomes of both APEC, UPEC and NMEC [5].

The similarity between the virulomes of APEC strains and human ExPEC emphasizes the potential threat of avian-associated zoonotic infections. Importantly, wild birds may act as a facilitator of the spread of APEC-associated virulence and antimicrobial resistance genes. For example, it has been shown that antibiotic resistance determinants can be transmitted from wild geese and swan strains of *Enterobacteriaceae* to strains in domestic birds, and from the latter to humans [6, 7].

The circumpolar regions of the Earth represent a unique geographical environment in which, despite extreme climatic conditions, the high productivity of shelf seas [8] maintains a high level of faunal biodiversity. The coasts of the Arctic and Southern Oceans within the continental Antarctic, Antarctic and sub-Antarctic archipelagos are points of attraction for billions of migrating birds, a significant part of which make long, including transcontinental flights. For example, the Palaearctic-African migration system alone includes 2.1 billion migrating individuals [9]. Colonies of migrating birds in high latitudes can be points of active intraspecies and interspecies contacts between birds and other animals, accompanied by the exchange of microbiota, including the pathogenic part of it [10].

In this regard, it seems important to study the distribution of bird-associated pathogens in ornithogenic ecosystems formed around bird colonies on the coasts of the Arctic and Antarctic seas.

At the same time, phylogeography and genetic features of such an actual object of epidemiologic and epizootologic surveillance as *E. coli* remains virtually unstudied in polar regions.

The aim of the study is the assessment of the pathogenic potential of *E. coli* strains distributed in the polar regions of the Earth based on the analysis of the genomes of these bacteria from a sample characterizing typical ornithogenic ecosystems of the Arctic and Antarctica.

Materials and methods

Collections of *E. coli* strains isolated from ornithogenic biological material (droppings, feces and carcasses of fallen birds, nest substrates, microbial mats of water bodies contaminated with bird droppings) during several expeditions were used in this study.

In particular, during the implementation of the scientific program of the Russian Arctic expedition on the Svalbard archipelago in 2018, 28 samples of ornithogenic material were collected, from which 6 isolates were isolated, in the expedition of Arctic Floating University (2023) — 8 isolates from 38 samples, in the 68th Russian Antarctic expedition of 2022–2023 — 19 isolates from 29 samples.

The present study describes the *E. coli* cultures isolated in the bird colonies of the Svalbard archipelago (West Spitsbergen Island), Novaya Zemlya and Franz Josef Land archipelagos, as well as on the islands of the Haswell Archipelago, which became one of the key ornithological territories of East Antarctica due to many thousands colonies of Adelie and Emperor penguins.

Furthermore, five *E. coli* strains isolated from cloacal flushes during bird ringing in the spring-summer period of 2023 at the Ladoga ornithological station (Nizhne-Svirsky Reserve, Gumbaritsy tract, Leningrad region) were used as comparison strains. All Arctic and Antarctic cultures were isolated without the use of enrichment methods using dense nutrient media when cultured directly in the field, as described previously [11].

The procedure of biological material sampling was carried out in accordance with generally accepted ethical normsю The research protocol was approved by the Ethics Committee of the which is confirmed by the decision of the Local Ethics Committee of North-Western State Medical University named after I.I. Mechnikov (Protocol No. 3, March 13, 2024).

Species identification of the isolated strains was performed using time-of-flight mass spectrometry (MALDI-TOF) on a Bactoscreen instrument (Litech). Mass spectra were analyzed using Biotyper 3.1 software.

As a result of random sampling, 16 strains were selected for whole-genome sequencing (WGS), followed by annotation and evaluation of pathogenic potential.

Information on the sources of cultures whose genomes were sequenced is presented in **Table 1**.

Biolabmix kits were used for genomic DNA isolation. Genomic sequencing was performed using BGI technology at the Pasteur Research Institute of Epidemiology and Microbiology. Genome annotation was performed using the RAST server (https://rast.nmpdr.org/rast.cgi), drug resistance and virulence genes were searched using the ABRicate v 0.8 program (https://github.com/tseemann/abricate), and the MEGARes (https://megares.meglab.org/amrplusplus/latest/html), Comprehensive Antibiotic Resistance Database, CARD 3.0.2 (https://card.mcmaster.ca/analyze/rgi) and VFDB (https://www.mgc.ac.cn/VFs/) databases were used for this purpose.

The antigenic structure of *E. coli* was determined using the online tool SerotypeFinder 2.0 (https://cge.food.dtu.dk/services/SerotypeFinder/). The MLST 2.0 resource (https://cge.food.dtu.dk/services/MLST/) was used to evaluate the results of multilocus sequencing-typing (MLST). The WGS typing results for bovine genes obtained using the online tool cgMLSTFinder 1.2 (https://cge.food.dtu.dk/services/cgMLSTFinder/) were compared with the data on the corresponding cgMLST types deposited in the EnteroBase database (https://enterobase.warwick.ac.uk/species/index/ecoli), allowing for differences (Max Number MisMatches) of no more than 20 single polymorphisms (SNPs).

Results

The annotation of genomes allowed us to determine their belonging to different STs in MLST and WGS-typing schemes. The main characteristics of the studied genomes and access numbers to their sequences are presented in **Table 2.**

Genes encoding AmpC-like beta-lactamases, the hyperproduction of which provides resistance to cephalosporins, were identified in all genomes studied [12]. In addition, the genome of the Arctic strain *E. coli* 33-1 was characterized by the presence of a plasmid of about 78,000 bp containing the gene for the extended-spectrum beta-lactamase TEM-1b and transposon Tn1721, which includes tetracycline resistance genes (*tetA-tetR*). Numerous pathogenicity factor genes associated with adhesion, invasion and iron capture were detected in the genomes of the studied microorganisms (**Table 3**).

The wide representation of ExPEC pathogenicity factor genes in the genomes of the strains under study raises the question of the potential association of these strains with cases of infectious diseases in humans.

Using the EnteroBase database (https://enterobase.warwick.ac.uk/species/index/ecoli), which accumulates global data on cgMLST genotyping results and currently includes information on more than 340,000 *E. coli* strains, a search was carried out for information on the geographical distribution of cgSTs identified in this study and their sources of isolation. The information was retrieved from the metadata provided in EnteroBase database for 11 STs (**Table 4**).

Discussion

In this study, an attempt was made to generate a sample of *E. coli* strains associated with high-latitude ornithogenic ecosystems typical of circumpolar regions in both the Northern and Southern Hemispheres.

In the Arctic, our studies focused on strains associated with marine colonial birds (Long-tailed Duck, Thick-billed Buzzards) and goose species (Bean Goose,

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Table 1. Characteristics of studied isolates

No.	Isolates	Place of isolation, coordinates	Source of isolation
		Isolates associated with bird ecosystems of the Arctic	
1	67Spits	Svalbard archipelago, Barentsburg, 78°03'N 14°16'E	Feces of *Rissa trydacyla*
2	70_2Spits	Svalbard archipelago, Gronfjorden Bay coast, N 78°00'36.2"N 14°18'09.7"E	Feces of *Rissa trydacyla*
3	89Spits	Svalbard archipelago, Barentsburg, 78°03'N 14°16'E	Feces of *Anser brachyrhynchus*
4	97Spits	Svalbard archipelago, Barentsburg, 78°03'N 14°16'E	Feces of *Anser brachyrhynchus*
5	AFU_2	Yugorsky Peninsula, White Nose Cape, 69°36'14.7"N 60°12'08.1"E	Feces of *Somateria mollissima*
6	AFU_32_1	Novaya Zemlya archipelago, Cape of Desire, 76°57'18.5"N 68°34'41.9"E	Feces of polar bear near the *Rissa trydacyla* bird spot
7	AFU_33_1	Novaya Zemlya archipelago, North Island, Cape of Desire, 76°57'18.5"N 68°34'41.9"E	Nests in the bird spot of *Rissa trydacyla*
8	AFU_43_1	Архипелаг Земля Франца-Иосифа, о-в Вильчека, 79°53'41.8"N 58°44'07.2"E Franz Josef Archipelago, Wilczek Island, 79°53'41.8"N 58°44'07.2"E	Egg shell of *Uria lomvia*
9	AFU_55_1	Franz Josef Archipelago, Komsomolskie islands (South Island), 80°34'48.3"N 58°32'40.2"E	Pond near the *Sterna paradisaea* bird spot
		Isolates associated with bird ecosystems of the Antarctic	
10	15myr	West Antarctica, Queen Mary Land, Haswell Archipelago, Haswell Island, 66°31'36.6"S 93°00'20.8"E	Adelie penguin (*Pygoscelis adeliae*), cloaca sample
11	17_1myr	West Antarctica, Queen Mary Land, Haswell Archipelago, Haswell Island, 66°31'36.6"S 93°00'20.8"E	Stomach sample from *Fulmarus glacialoides*
12	28myr	West Antarctica, Queen Mary Land, Haswell Archipelago, Tokareva Island, 66°32'06.1"S 92°58'25.8"E	Feces of *Adelie penguin* (*Pygoscelis adeliae*)
		Isolates form birds in European region of Russia, Ladoga Ornithological Station (LOS)	
13	LOS_49	Nizhne-Svirskiy Reserve, LOS, 60°40'35.0"N 32°56'27.2"E	Cloaca sample from *Poecile palustris*
14	LOS_51	Nizhne-Svirskiy Reserve, LOS, 60°40'35.0"N 32°56'27.2"E	*Anas crecca* feces
15	LOS_52	Nizhne-Svirskiy Reserve, LOS, 60°40'35.0"N 32°56'27.2"E	Cloaca sample from *Turdus pilaris*
16	LOS_54	Nizhne-Svirskiy Reserve, LOS, 60°40'35.0"N 32°56'27.2"E	Cloaca sample from *Turdus pilaris*

Eiders). These bird species differ both in the ecological niches they occupy and in the duration and directions of migration, which may influence the structure of their microbiota, determining the probability of colonization by different *E. coli* genotypes.

At the same time, it is necessary to take into account the possibility of forming a single reservoir for populations of this microorganism along the entire coast of the Arctic Ocean, determined by bird migrations in the meridional direction. Thus, it was recently found out that a significant part of the kittiwake population migrates from the South Island of Novaya Zemlya to the wintering grounds on the coast of the North Pacific Ocean [13]. The spread of pathogens with Arctic migratory birds (predominantly from the goose group) simultaneously in latitudinal and meridional directions was previously shown for influenza viruses [14]. It should be noted that bean geese, the strains from which were studied in this research, make seasonal migrations from the Svalbard archipelago to wintering grounds in Belgium and the Netherlands, and against the background of global climate change in the Arctic, these birds are actively exploring Novaya Zemlya as well [15].

Three Antarctic strains, whose genomes were characterized in the present study, were isolated in the territory of Adelie penguin colonies on the islands of the Haswell Archipelago in East Antarctica. In spite of the fact that penguins of this species are endemic to Antarctica, in the territories of the colonies they neighbor also with long-distance migratory species of birds. For example, a frequent inhabitant of the Haswell Archipelago, the south polar skua, is able to make long seasonal migrations and reach the North Pacific and the North Atlantic [16]. Thus, the populations of Antarctic birds are not isolated from the global circulation of pathogens, as evidenced, in particular, by the circula-

Table 2. Genetic characteristics of isolated *E. coli* strains

Strain	Region of isolation	Serotype	Sequence type (ST)	ST by core genome (cgST)	GenBank access number
67Spits	Arctic	O4:H5	12	10054	JAYEAG010000000
70_2Spits	Arctic	O43:H2	937	28072	JAYEAE000000000.1
89Spits	Arctic	O83:H1	135	87221	JAYEAD000000000.1
97Spits	Arctic	O166:H49	1246	162	JAYEAC000000000.1
AFU_2	Arctic	O93:H16	8097	132840	JAYEAJ000000000
AFU_32_1	Arctic	O54:H45	491	11903	JAYEAI000000000
AFU_33_1	Arctic	O15:H2	69	189219	JAYEAH000000000
AFU_43_1	Arctic	O9:H49	6163*	1429	JBIQXY000000000
AFU_55_1	Arctic	O39:H4	1155	196482	JBIQXZ000000000
15myr	Antarctic	O8:H7	127	196780	JBIQXV000000000
17_1myr	Antarctic	O6:H31	196	133718	JBIQXW000000000
28myr	Antarctic	O182:H38	1632	94237	JBIQXX000000000
LOS_49	European Russia	O8:H5	2594*	47119	JBIQYA000000000
LOS_51	European Russia	O85:H8	297	114487	JBIQYB000000000
LOS_52	European Russia	N/i	1333*	119313	JBIQYC000000000
LOS_54	European Russia	N/i	58	126100	JBIQYD000000000

Note. *Genotypes with single nucleotide polymorphisms in genes for which sequencing-typing was performed, distinguishing them from the specified sequence types. N/I — not identified.

tion in the Antarctic of influenza virus strains identical to those isolated in other geographical regions [17].

Analysis of the geographical distribution of ST *E. coli* determined by the cgMLST method demonstrated their cosmopolitanism, which was manifested by the detection of identical cgST in geographically distant regions of the planet. For example, cgST 133718 was detected in Antarctica (strain 17_1myr) and Great Britain, and cgST 11903, to which strain 32-1 from the northernmost point of the New Earth belonged, was previously detected in the USA.

Despite the fact that all the strains studied belong to different genetic lineages (ST and serotypes), they are all united by the presence of an extensive virulome. As shown in Table 3, all strains from polar regions have a set of virulence factors that allow them to be considered as potential agents of human infections. In this respect, in general, they do not differ from the strains isolated from birds in the Leningrad Region.

Thus, 10 out of 12 polar strains contained genes or combinations of genes determining the synthesis of hemolysins A, E and F, which together with siderophores of the enterobactin, aerobactin and yersinibactin clusters participate in iron capture during the infection process [18]. It should be noted that the genes of the yersinibactin operon, found in half of the Arctic and Antarctic cultures, are part of the high pathogenicity island. This mobile genetic element is associated, as previously shown, with virulent UPECs [19].

The identified virulence factors for which localization in mobile genetic elements has been described include the uropathogenicity protein. The importance of this factor in damaging mammalian cells has been demonstrated, which is essential in the development of urinary tract infections [20].

In the studied sample, strains marking the enteroaggregative *E. coli* (EAEC) pathotype were also identified, in particular, the eilA virulence regulator gene (Salmonella HilA homolog) and the enteroaggregative protein gene (air), common in EAEC, were detected in the genomes of strains AFU-33-1 and AFU-43-1 from Novaya Zemlya and Franz Josef Land. The strain AFU-33-1 belongs to ST 189219 according to cgMLST results, which is widely represented in a number of European countries, USA and Brazil as a pathogen of generalized human infections. Thus, the spread of a peculiar epidemic clone of *E. coli* was observed in territories where there were no permanent human settlements.

In general, the spread of global genetic lineages of *E. coli* in high latitudes is consistent with the notion that the Rapoport Rule is observed for human pathogens, which states that as one moves from the equator to the poles, the distribution ranges of species or other taxonomic groupings increases [21].

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Table 3. Pathogenic determinants in studied *E. coli* strains

Pathogenic determinants	17myr	15myr	28myr	67Spits	70_2Spits	89Spits	97Spits	AFU_2	AFU_32_1	AFU_33_1	AFU_43_1	AFU_55_1	LOS_49	LOS_51	LOS_52	LOS_54
Hemolysins																
Hemolysin A (hlyA)	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Bird hemolysin E (hlyE)	–	+	+	–	+	–	+	+	–	+	+	–	–	+	–	+
Hemolysin F (hlyF)	+	+	–	–	–	–	–	–	–	+	–	+	+	–	–	+
Siderophores																
Enterobactin operon	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+
Yersiniabactin operon	+	–	–	+	–	+	–	–	+	+	–	+	–	+	+	–
Aerobactin operon	+	+	–	–	–	–	–	–	–	+	–	+	–	–	–	–
Toxins																
Heat-stable enterotoxin EAST-1	–	–	–	–	–	–	+	–	+	–	–	–	–	–	+	+
Subtilase	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Cytolethal distending toxin (cdt)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Adhesion, invasion and biofilm formation determinants																
Adhesin AIDA-I	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	+
Aggregation substance Tia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Gen *eilA* homologue (*Salmonella* HilA homolog)	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
Enteroaggregative protein (air)	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
Factor of invasion of brain endothelium (ibeA)	–	–	–	–	–	+	–	–	–	–	–	+	+	–	+	–
Serum survival gene (iss)	+	–	+	–	+	–	+	–	–	+	–	+	+	–	–	+
Arylsulfatase AslA	+	–	+	+	–	+	–	–	–	–	–	–	+	–	+	–
Heat-resistant agglutinin (hra)	+	–	–	+	+	–	+	–	–	–	–	–	–	–	–	+
Cytotoxic necrotizing factor (cnf)	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Uropathogenic specific protein (usp)	+	–	–	+	–	+	–	–	–	–	–	+	–	–	–	–
Curlin CsgA	+	+	+	+	+	+	+	+	+	–	+	+		+	+	+
Homologue of the *Shigella flexneri* SHI-2 pathogenicity island gene *shiA*	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	+
Intimin-like adhesin FdeC	+	+	–	+	+	+	+	+	+	–	+	–	–	–	+	+
Serine protease autotransporters of *Enterobacteriaceae* (SPATE)	+	–	–	+	–	+	–	–	+	+	–	+	+	–	+	–

The search for antimicrobial resistance genes in the genomes of *E. coli* strains carried out in this study indicates the absence (within our sample) of critical determinants of drug resistance. Nevertheless, the presence of genetic determinants of resistance to cephalosporins and tetracycline in the genome of the Arctic strain AFU 33-1 indicates the possibility of circulation of mobile genetic elements carrying these genes in the wild and their preservation in the microbiome of Arctic animals in the absence of antibiotic pressure.

Conclusion

The results of the study indicate the circulation of *E. coli* strains with strong pathogenic potential in high-latitude Arctic and Antarctic ornithogenic ecosystems. The analysis of the genomic data indicates the presence of genetic lineages that are geographically widespread in these regions, highlighting the importance of monitoring for epidemic clones of *E. coli*, together with monitoring for other pathogens, in high-latitude bird colonies.

Table 4. Geographical distribution and isolation sources of sequence types (cgST) of the identified strains

Sequence type by core genome (cgST)	Region (regions) of isolation	Source of isolation	Reference number of the Sequence Read Archive (SRA) for strains most similar to the identified STs
10054	Europe, Anand Islands	Human (blood culture)	ERR434967
28072	USA	Livestock (cows)	SRR3972294
162	Australia	*Chroicocephalus novaehollandiae*	SRR24017969
132840	USA	Livestock (calves)	SRR26082289
11903	USA	Human (patient with urinary tract infection)	SRR1314409
189219	USA, Spain, Brazil, Denmark, UK	Chicken, human (blood culture and urine during urinary tract infection)	SRR17774295, ERR13306341, ERR4014451, SRR21849316 SRR21846782
196780	USA	Livestock (cows)	SRR19171807
133718	UK	Wild birds (Anseriformes)	SRR11410512
114487	USA	Beef	SRR10156198
119313	Netherlands	Human (blood culture)	ERR3650458
126100	Sweden	Poultry	SRR14477383

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Original Study Article
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Protective potential of structural proteins of the SARS-CoV-2 virus in protecting against COVID-19

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Abstract

Introduction. Many different vaccines for the prevention of COVID-19 have received emergency use approval in the shortest possible time. Due to the high rate of variability of the pathogen, in this study we analyzed the variability of the structural proteins of the SARS-CoV-2 virus and compared their protective potential in protecting animals from COVID-19.

The aim of the study was to compare the protective potential of the SARS-CoV-2 structural proteins in protecting animals from COVID-19.

Materials and methods. The SARS-CoV-2 virus was used in the study. Transgenic mice B6.Cg-Tg(K18-ACE2)2Prlmn/J (F1) were used as model animals. Recombinant adenoviral vectors rAd5-S, rAd5-N, rAd5-M were used for immunization of animals. Various genetic, virological and immunological methods, as well as methods of working with animals, were used in the study.

Results. The largest number of amino acid substitutions in the structural proteins of different SARS-CoV-2 variants was detected in glycoprotein S, the smallest — in nucleoprotein N. In the COVID-19 animal model, it was shown that only the use of glycoprotein S as a vaccine antigen allows to form protective immunity that protects 100% of animals from a lethal infection caused by the SARS-CoV-2 virus, while the use of protein N protects 50% of animals from a lethal infection, and protein M does not have a protective potential.

Conclusion. The data obtained, as well as the analysis of the epidemiological efficacy of various mRNA and vector vaccines, demonstrate that the use of the SARS-CoV-2 glycoprotein S as an antigen allows to form the highest level of protection. Due to the constant change in circulating variants of the SARS-CoV-2 virus, the decrease in the effectiveness of the vaccines with the original antigen composition against new variants of the virus and the continuing high incidence of COVID-19, it is necessary to continuously monitor the effectiveness of vaccines against new variants of the virus and promptly update the antigen composition of vaccines when a decrease in effectiveness is detected.

Keywords: *antigen, SARS-CoV-2, protective immunity, COVID-19*

Ethical approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Biomedical Ethics Committee of the N.F. Gamaleya National Research Center for Epidemiology and Microbiology (Protocol No. 24 of April 21, 2022).

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Протективный потенциал структурных белков вируса SARS-CoV-2 в защите от COVID-19

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Аннотация

Введение. Множество различных вакцин для профилактики COVID-19 в кратчайшие сроки получили разрешение на экстренное применение. В связи с высоким уровнем изменчивости возбудителя, важно учитывать вариабельность структурных белков вируса SARS-CoV-2 и их протективный потенциал в защите животных от COVID-19.

Цель исследования — сравнить протективный потенциал структурных белов вируса SARS-CoV-2 в защите животных от COVID-19.

Материалы и методы. В качестве модельных животных при исследовании вируса SARS-CoV-2 служили трансгенные мыши B6.Cg-Tg(K18-ACE2)2Prlmn/J (F1). Для иммунизации животных использовали препараты рекомбинантных аденовирусных векторов: rAd5-S, rAd5-N, rAd5-M. В работе применяли различные генетические, вирусологические и иммунологические методы, а также методы работы с животными.

Результаты. Наибольшее количество аминокислотных замен в структурных белках разных вариантов SARS-CoV-2 было обнаружено в гликопротеине S, наименьшее — в нуклеопротеине N. На модели COVID-19 у животных показано, что только использование гликопротеина S в качестве антигена в составе вакцинного препарата позволяет сформировать протективный иммунитет, который защищает 100% животных от летальной инфекции, вызванной вирусом SARS-CoV-2, при этом использование белка N позволяет защитить 50% животных от летальной инфекции, а белок M не обладает протективным потенциалом.

Заключение. Полученные данные, а также анализ данных эпидемиологической эффективности разных мРНК- и векторных вакцин демонстрируют, что использование гликопротеина S вируса SARS-CoV-2 в качестве антигена позволяет сформировать наиболее высокий уровень защиты. Учитывая постоянную смену циркулирующих вариантов вируса SARS-CoV-2, снижение эффективности используемых вакцин с исходным антигенным составом в отношении новых вариантов вируса и сохраняющийся высокий уровень заболеваемости COVID-19, необходимо проводить непрерывный мониторинг эффективности вакцинных препаратов в отношении новых вариантов вируса и своевременно проводить актуализацию антигенного состава вакцин при выявлении снижения эффективности.

Ключевые слова: *антиген, SARS-CoV-2, защитный иммунитет, COVID-19*

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Introduction

Since the identification of the causative agent of COVID-19, the SARS-CoV-2 virus, vaccine development has proceeded at an unprecedented and extraordinary pace thanks to the collaborative efforts of researchers, manufacturers and governments around the world. This has enabled the vaccines to be quickly approved for emergency use and introduced into civilian circulation to vaccinate populations around the world.

SARS-CoV-2 virus belongs to single-stranded (+) RNA-containing viruses of the *Betacoronavirus* genus of the *Coronaviridae* family, the genome size varies from 29.8 to 29.9 thousand nucleotides, virions have a spherical or ellipsoidal shape with an average diameter of 108 ± 8 nm and contain 4 major structural proteins. The outer surface of the virion is covered with a surface protein, glycoprotein S, while the virus outer membrane contains membrane protein M and envelope protein E. Inside the virion is a ribonucleoprotein complex consisting of nucleocapsid protein (N) and virus RNA [1]. The surface glycoprotein S of SARS-CoV-2 virus is a trimer with a molecular mass of about 600 kDa. Located on the virion envelope, it plays a key role in viral infection through receptor recognition and subsequent fusion of the virus and host cell membranes. Protein S has been shown to elicit a robust immune response, making it a dominant target for the development of vaccines to prevent COVID-19 [2-5]. The major protein component within the virion is the nucleocapsid protein N, which is essential for binding and packaging genomic RNA into a ribonucleoprotein complex (RNP complex) within the virion [6]. Coronavirus membrane protein (M) is the most abundant viral structural protein and plays a central role in virus assembly and morphogenesis [7]. The M and N proteins could potentially serve as targets for inclusion in candidate vaccine for COVID-19 prevention [8]. In addition to these structural proteins, SARS-CoV-2 encodes 16 non-structural proteins (nsp1-16) and 9 accessory proteins.

To date, about 50 vaccines for COVID-19 prevention have been registered[1]. The most widely used vaccines are those developed on 4 technological platforms: vector vaccines, mRNA vaccines, inactivated vaccines and subunit vaccines. Vector and mRNA vaccines are the most effective in protecting against COVID-19 [9]. Most vaccines contain the key protective antigen – surface glycoprotein S of the SARS-CoV-2 virus. Immunization with such vaccines allows the formation of a full immune response to the protein [10]: both humoral immune response (preventing the virus from entering the cell) and cellular cytotoxic immune response (necessary for the elimination of infected cells) are formed [11–15]. Immunization with inactivated vaccines induces a weaker humoral immune response and almost no T-cell immune response. In this case, antibodies of IgG, IgM and IgA classes are formed to various SARS-CoV-2 proteins, not only to S [16, 17]. The issue of immune correlates of protection against COVID-19 is still being studied, and a direct correlation between the level of neutralizing antibodies and protection against the disease has been shown [18]. The majority of epitopes for neutralizing antibodies are located in the region of the receptor-binding domain and the N-terminal domain of glycoprotein S [19].

SARS-CoV-2 virus has been actively evolving since its entry into the human population [20]. During the 4 years of the COVID-19 pandemic, there was a constant change of circulating variants of the pathogen, which has been accompanied by an increase in disease incidence. The most significant wave of desease was registered in early 2022 and was associated with the spread of the first sublineages (BA.1 and BA.2) of the Omicron variant of the SARS-CoV-2 virus[2]. The emergence and spread of new Omicron variant sublineages (BA.1, BA.5, XBB, BA.2.86, etc.) were accompanied by an increase in disease incidence around the world, including among the vaccinated population, therefore, it is critical to conduct continuous monitoring of the efficacy of vaccines against circulating variants for rapidly change of antigenic composition of vaccines[3].

In the Russian-language literature, there are no data on comparative analysis of the protective efficacy of different SARS-CoV-2 virus antigens. These data will allow adequate assessment of the need to include different antigens in the composition of vaccines for COVID-19 prevention for the greatest efficacy against different variants of SARS-CoV-2 virus.

Given the high level of variability of the virus, **the aim** of this study was to compare the protective potential of structural proteins of SARS-CoV-2 virus in the protection of animals against COVID-19. To achieve this goal, the following objectives were set: to analyze the variability of structural proteins of SARS-CoV-2 virus, to study the protective efficacy of different struc-

[1] COVID-19 Vaccine tracker. Approved vaccines.
URL: https://covid19.trackvaccines.org/vaccines/approved/

[2] NextStrain. Genomic epidemiology of SARS-CoV-2 with subsampling focused globally since pandemic start.
URL: https://nextstrain.org/ncov/gisaid/global/all-time
WHO. WHO COVID-19 dashboard. URL: https://data.who.int/dashboards/covid19/cases?m49 = 001

[3] OurWorldInData. United States: COVID-19 weekly death rate by vaccination status, All ages.
URL: https://ourworldindata.org/grapher/united-states-rates-of-covid-19-deaths-by-vaccination-status
WHO. Statement on the antigen composition of COVID-19 vaccines. 18.05.2023.
URL: https://www.who.int/news/item/18-05-2023-statement-on-the-antigen-composition-of-covid-19-vaccines
WHO. Statement on the antigen composition of COVID-19 vaccines. 26.04.2024.
URL: https://www.who.int/news/item/26-04-2024-statement-on-the-antigen-composition-of-covid-19-vaccines

DOI: https://doi.org/10.36233/0372-9311-577

tural proteins of SARS-CoV-2 virus on the COVID-19 laboratory animals model.

Materials and methods

Virus

SARS-CoV-2 virus was obtained from the State Virus Collection of N.F. Gamaleya NRCEM: Wuhan B.1.1.1 hCoV-19/Russia/Moscow_PMVL-1/2020. All studies with viable SARS-CoV-2 virus were conducted in BSL-3 laboratory according to SanPiN 3.3686-21 "Sanitary and Epidemiologic Requirements for the Prevention of Infectious Diseases". SARS-CoV-2 virus production was carried out in Vero E6 cell culture. The infectious virus titer was determined on Vero E6 cell culture by 50% tissue culture infectious dose 50 ($TCID_{50}$). The $TCID_{50}$ titer was calculated using the Spearman–Kerber method.

Mammallian cell lines

Vero E6 cell culture (African green monkey kidney epithelial cells) was obtained from the Laboratory of Cellular Microbiology of the N.F. Gamaleya NRCEM.

Animal models

The study used F1 transgenic mice obtained from crossing transgenic males B6.Cg-Tg(K18-ACE2)2Prlmn/J (Jackson Laboratory, https://www.jax.org/strain/034860; health status SOPF) and non-transgenic females C57BL/6 Gamrc (N.F. Gamaleya NRCEM; health status SPF), hereinafter referred to as hACE2-transgenic mice. Female hACE2-transgenic mice weighing 18–20 g were used in the study. All animal studies were approved by the Biomedical Ethics Committee of the N.F. Gamaleya NRCEM (protocol No. 24 dated 21/04/2022). Expression of the *hACE2* gene in F1-generation C57BL/6 Tg(K18-ACE2)2Prlmn mice was confirmed by real-time polymerase chain reaction according to the Jackson Laboratory protocol for this line of mice [21].

Work with animal models

Animals were housed and handled in accordance with laboratory animal husbandry requirements[4]. Laboratory animals (n = 40) were housed in conventional cages for immunization; for experiments involving SARS-CoV-2 virus, animals were housed in the IsoCage N system (Tecniplast). The animals had free access to food and water.

Immunization and virus challenge of animals

The study used recombinant viral vectors based on human adenovirus type 5 (rAd5) carrying genes of structural proteins of SARS-CoV-2 virus of Wuhan B.1 variant: rAd5-S (carries the gene of glycoprotein S), rAd5-N (carries the gene of nucleoprotein N), rAd5-M (carries the gene of membrane protein M) were used for immunization of animals. Animals of the groups (n = 10 per group) that received the vaccine were injected with rAd5 vaccines at a dose of 10^9 v.p./animal intramuscularly twice with an interval of 21 days. Animals in the control group (n = 10) were injected with an equivalent volume of sterile buffer solution. One week after the 2nd immunization, animals were infected intranasally with SARS-CoV-2 virus at a dose of 10^5 $TCID_{50}$ and weight dynamics and survival rate were evaluated daily for 14 days after infection.

Organ sampling and viral load determination

Animals of all study groups (n = 4 per group) were euthanized on the 4th day after infection with an increased dose of inhalation anesthetic followed by cervical dislocation. Postmortem examination of the animals was performed, and lungs were sampled for macroscopic and viral load analysis. The selmpled organs were washed with saline and 10% homogenate was prepared using the MPbio FastPrep-24 device. The homogenates were centrifuged at 12,000g for 10 min and the supernatant was used for further analysis. The infectious titer of the virus was determined on Vero E6 cells according to the method described above.

Statistical and bioinformatics methods

Statistical processing of the research results was performed using the GraphPad Prism 10.2.3 computer program. The Student's t-test was used to analyze the data [22]. The covSPECTRUM online database was used to analyze the amino acid sequences of structural proteins of SARS-CoV-2 virus[5]. On the cov-spectrum.org portal in the Compare variants mode we selected the sequence of the desired protein comparison of new variants of SARS-CoV-2 virus[6] in the Amino acid

[4] Руководство по лабораторным животным и альтернативным моделям в биомедицинских технологиях / под ред. Н.Н. Каркищенко, С.В. Грачева. М.; 2010. 354 с.

[5] covSPECTRUM. Detect and analyze variants of SARS-CoV-2. URL: https://cov-spectrum.org/

[6] covSPECTRUM. B.1.617.2 (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=B.1.617.2&
covSPECTRUM. BA.1 (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=ba.1&
covSPECTRUM. BA.5 (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=ba.5&
covSPECTRUM. XBB (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=xbb&
covSPECTRUM. BA.2.86 (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/

changes section and performed a pairwise with the original virus variant[7]. To calculate the variability of amino acid composition, the number of detectable amino acid substitutions was divided by the total number of amino acid substitutions and demonstrated as a percentage.

Results

Variability of structural antigens

We conducted a bioinformatics analysis of amino acid sequences of structural proteins of SARS-CoV-2 virus of different variants that circulated in Russia from March 2020 to July 2024 (**Table**). Among the 4 analyzed proteins, the largest number of amino acid substitutions was detected in glycoprotein S, the smallest – in nucleoprotein N. Thus, in variant KS.1, which has been circulating in Russia since spring 2024, 64 amino acid substitutions were detected in glycoprotein S and 8 in nucleoprotein N.

Variability of amino acid composition of structural proteins of SARS-CoV-2 virus circulating in Russia in 2020–2024

SARS-CoV-2 variant	Variability of amino acid composition, % relative to the original virus variant			
	S	N	M	E
Delta	0,79	0,59	0,45	0,00
Omicron BA.1	2,83	0,88	1,35	1,33
Omicron BA.5	2,67	1,03	1,35	1,33
Omicron XBB	3,22	1,03	0,90	2,67
Omicron BA.2.86	4,63	1,17	2,25	1,33
Omicron KS.1	5,03	1,17	2,25	1,33

Source: covSPECTRUM online database.

Protective efficacy of SARS-CoV-2 virus structural proteins in a lethal infection model in hACE2-transgenic mice

In order to compare the protective efficacy of structural proteins of SARS-CoV-2 virus, we obtained recombinant viral vectors based on human adenovirus serotype 5 carrying genes of glycoprotein S, nucleoprotein N or membrane protein M. The study was performed on hACE2-transgenic mice. Animals were immunized twice at 21-day intervals and 7 days after the 2nd immunization, animals were infected intranasally with SARS-CoV-2 (Wuhan-like) virus at a dose of 10^5 $TCID_{50}$ per animal. The effectiveness of antigens in protection against infection was analyzed by several parameters: lethality, severity of the course of infection (weight loss) and reduction of viral load in the lungs of vaccinated animals compared to controls.

Survival analysis showed that only the use of glycoprotein S as antigen can protect 100% of animals from lethal infection caused by SARS-CoV-2 virus (**Fig. 1**, *a*). Analysis of the severity of the course of infection also demonstrated that only the use of glycoprotein S can protect 100% of animals from infection (Fig. 1, *b*).

On the 4th day after infection, some animals were euthanized for macroscopic analysis of lung condition and viral load analysis. Analysis of the lung condition on the 4th day after infection showed that the most pronounced damage was found in the group of control animals and the group of animals that received the vaccine based on protein M (leukocytic infiltration, atelectasis and changes in membrane structures of the alveolar wall were observed). Less pronounced damage was detected in animals in the group that received the vaccine based on nucleoprotein N. Animals that received the vaccine based on glycoprotein S had no lung damage (**Fig. 2**).

When analyzing the viral load in the lungs of infected animals in the control group and in the groups that received vaccines based on nucleoprotein N and membrane protein M, viable virus was detected. The animals that received the vaccine based on nucleoprotein N showed a significant decrease in viral load by 1.3 log10 $TCID_{50}$. In the group that received a vaccine based on glycoprotein S no viable SARS-CoV-2 virus was detected and the viral load decreased by 5 lg $TCID_{50}$ (**Fig. 3**).

Discussion

When selecting an antigen for inclusion in candidate vaccines, it is important to have an understanding of the immunologic features of the response to natural COVID-19 infection. The interaction of immune cells with the major structural proteins of the virus induces the formation of an antiviral immune response. For SARS-CoV-2 virus, these structural proteins are S, M, N and E. Despite the immunogenicity of glycoprotein S, nucleoprotein N and membrane protein M also contribute significantly to the development of a specific immune response. In patients with COVID-19, specific antibodies to protein N are detected early, whereas antibodies to glycoprotein S are detected 4-8 days after the onset of disease symptoms, which is probably due to the highest representation of nucleoprotein N in the virion [23, 24]. The cellular immune response also plays an important role in protection against COVID-19. A number of studies have shown that active proliferation of $CD4^+$ and $CD8^+$ T cells correlates with a less

from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=ba.2.86&
covSPECTRUM. KS.1 (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=ks.1&

[7] covSPECTRUM. B (Nextclade).
URL: https://cov-spectrum.org/explore/World/AllSamples/from%3D2020-01-01%26to%3D2024-10-11/variants?nextcladePangoLineage=B&nextcladePangoLineage1=B.1&

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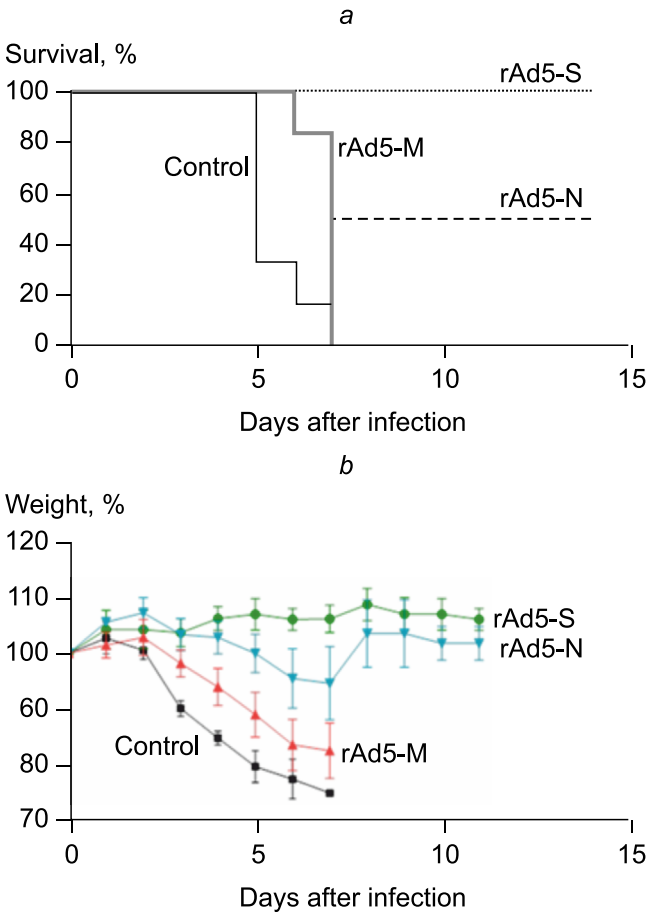

Fig. 1. Survival (*a*) and weight dynamics (*b*) of hACE2-transgenic mice from immunized and control groups (n = 10, days 0–4; n = 6, days 5–14) after infection with the SARS-CoV-2 virus.

The mean and standard deviation for each time point are shown in the figure (*b*).

severe course of the disease and a high degree of virus elimination[8]. The cellular immune response is also highly specific to SARS-CoV-2 structural proteins. Determination of the SARS-CoV-2 T-cell epitopes, including 21 studies, showed that of the total number of $CD4^+$ epitopes analyzed, 33% belonged to protein S, 11% to protein N, and 10% to protein M; of the total number of $CD8^+$ epitopes analyzed, 26% belonged to protein S, 7% to protein N, and 6% to protein M [25]. A polyfunctional N-specific $CD8^+$ T-cell response is associated with milder COVID-19 disease severity [26]. Because N is conserved between different SARS-CoV-2 variants, N-specific $CD4^+$ T cells could potentially provide protection against different genetic variants of SARS-CoV-2 [27].

Nowadays, most vaccines for COVID-19 prevention used in clinical practice are based on glycoprotein S of the SARS-CoV-2 virus. However, there is still debate about which antigen should be included in COVID-19 vaccines. This is due to the high variability of glycoprotein S and the decreased efficacy of existing vaccines based on it against new SARS-CoV-2 virus variants.

When analyzing the amino acid sequences of structural proteins of SARS-CoV-2 virus — variants from Delta to current KS.1 — we demonstrated that the largest number of substitutions was found in glycoprotein S, the smallest — in nucleoprotein N, which makes protein N the most conservative among structural proteins of SARS-CoV-2 virus.

In order to directly compare the protective potential of SARS-CoV-2 virus structural proteins most represented in the virion, we obtained candidate vaccines based on recombinant human adenoviruses of serotype 5 carrying SARS-CoV-2 virus structural proteins: rAd5-M, rAd5-N and rAd5-S. These vaccines were used for immunization of hACE2-transgenic mice, after which the animals were infected with SARS-CoV-2 B.1.1.1 virus to evaluate the protective efficacy. Membrane protein M showed no protective efficacy — all immunized animals died after infection, while viable virus was detected in the lungs on the 4th day after infection at a titer similar to that of control animals. Similar data were obtained by J. Chen et al. when studying the effectiveness of plasmid DNA-based vaccines [28]. The use of nucleoprotein N as an antigen reduces the viral load in the lungs of immunized animals, but the reduction does not reach the required 2 $\log_{10}$; at the same time, pathological lesions were detected in the lungs of vaccinated animals, and protection against lethal infection caused by SARS-CoV-2 virus was only 50%, which correlates with the studies of other authors [29–32]. The use of glycoprotein S as an antigen made it possible to immunize animal models (no pathologic lesions and viable virus in the lungs) and protect all of them from lethal infection caused by SARS-CoV-2 virus, which is confirmed by the studies of other authors [33–36]. J. Chen et al. demonstrated that joint immunization with two DNA-based vaccines carrying S and N protein genes induced a more pronounced cellular and humoral immune response and had a greater protective efficacy against SARS-CoV-2 virus in a mouse model of infection [32]. R.L. Hajnik et al. showed on the COVID-19 hamster model that immunization with mRNA-S+N combined preparation induces a more pronounced protective response against SARS-CoV-2 virus veriants Delta and Omicron compared to single-component preparations [37]. Based on the above, the possibility of combining antigens in vaccine formulations for COVID-19 prevention should be considered in future studies. However, it should be taken into account that the inclusion of several antigens in vaccines significantly increases the cost of the production process and, consequently, the cost of the vaccine.

Analysis of the efficacy data of different vaccines for COVID-19 prevention in controlled clinical trials

[8] CDC. CDC Museum COVID-19 timeline. Centers for Disease Control and Preventio. URL: https://www.cdc.gov/museum/timeline/covid19.html

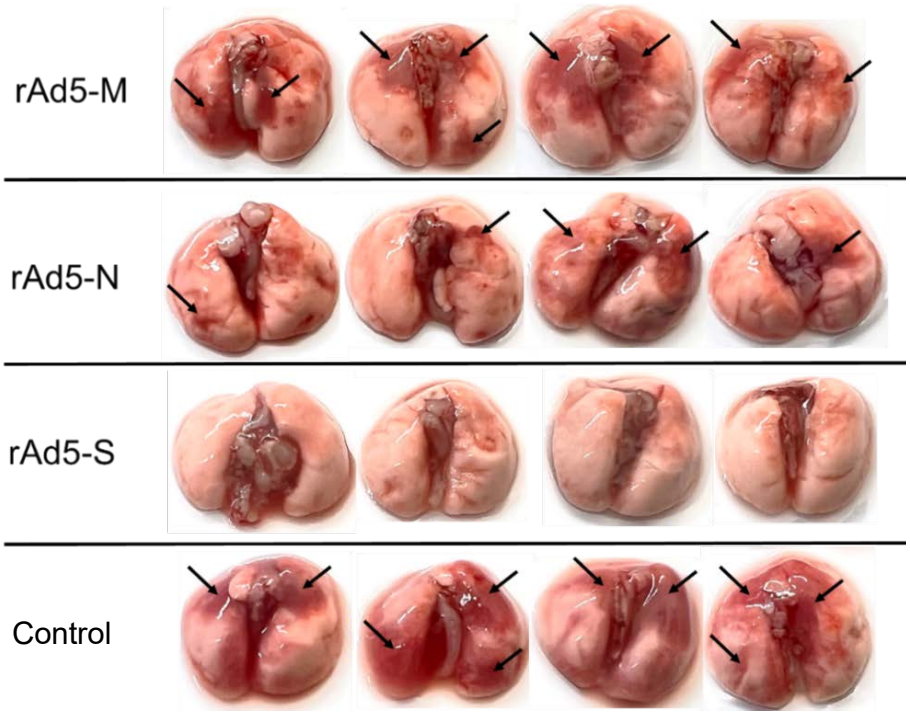

Fig. 2. Photographs of the lungs of hACE2-transgenic mice from the immunized and control groups (n = 4) on day 4 after infection with the SARS-CoV-2 virus. Arrows indicate areas of lung tissue damage.

worldwide showed that mRNA and vector vaccines carrying the glycoprotein S gene of SARS-CoV-2 virus allow to provide the highest level of population protection in terms of morbidity, hospitalization and COVID-19-associated deaths [9, 38]. Given the constant change of circulating SARS-CoV-2 virus variants, the decreasing efficacy of the vaccines used against new virus variants [39] and the continuing high incidence of COVID-19, it is necessary to continuously monitor the efficacy of vaccines against new virus variants. If a decrease in efficacy is detected, the antigenic composition of vaccines should be updated. These studies are harmonized with the WHO studies, based on the results of which, starting from 2022, WHO issues recommendations on changing the antigenic composition of vaccines[9]. In 2023, based on the results of efficacy monitoring, the antigenic composition of Russian vaccines of Gam-COVID-Vac line (vector vaccines carrying the gene of glycoprotein S of SARS-CoV-2 virus) was updated for XBB sublineage. Clinical trials of vaccines with updated composition showed a favorable safety profile, formation of neutralizing antibodies against Omicron sublineages circulating in 2023 and circulating in the first half of 2024, and today the vaccines have been introduced into civil circulation to protect the population against current circulating variants of SARS-CoV-2.

[9] WHO. Technical Advisory Group on COVID-19 Vaccine Composition. URL: https://www.who.int/groups/technical-advisory-group-on-covid-19-vaccine-composition-(tag-co-vac)

Conclusion

SARS-CoV-2 virus actively mutates, which leads to the emergence of new virus variants. Among the structural proteins, the one subject to the greatest variability is surface glycoprotein S, which plays an important role in the virus life cycle — internalization, and is also a key target for neutralizing antibodies.

Comparative analysis of the protective potential of different structural proteins of SARS-CoV-2 virus on the animal model of lethal infection showed that only the use of glycoprotein S allows to form a protective

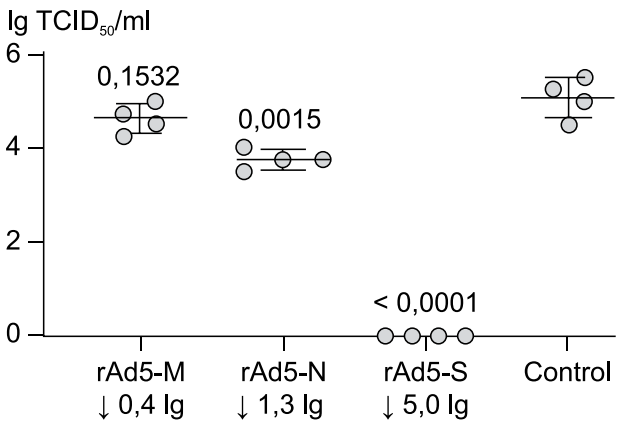

Fig. 3. Viral load in the lungs of hACE2-transgenic mice from the immunized and control groups (n = 4) on day 4 after infection with the SARS-CoV-2 virus.

The figure shows individual data for each animal, the arithmetic mean and standard deviation, as well as the significance level p (Student's t-test).

immune response that protects 100% of animals from lethal infection caused by SARS-CoV-2 virus, while the reduction of viral load in the lungs of animals on the 4th day after infection amounted to 5.0 $\log_{10}$ $TCID_{50}$ (100 000 times). At the same time, the use of nucleoprotein N resulted in a decrease in viral load of 1.3 $\log_{10}$ $TCID_{50}$ (20-fold), and the level of protection against lethal infection was at 50%.

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A clinical study of the immunogenicity and protective potency of a live recombinant GamLPV vaccine for intranasal use for the prevention of whooping cough in adult volunteers

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Abstract

Introduction. The increase in incidence rate of pertussis worldwide, short-term insufficient immunity induced by acellular pertussis vaccines (TDaP) and their failure to provide antibacterial protection and to prevent transmission of infection in human population dictate the development of new pertussis vaccines. A new live recombinant pertussis vaccine for intranasal use (GamLPV) has completed preclinical studies in experiments on nonhuman primates and 2 phases of clinical trials involving adult healthy volunteers, in which the safety, immunogenicity and protective activity of the GamLPV were proven. Method and scheme of vaccine administration have been worked out.

Aim. Confirmation of the immunogenicity and protective antibacterial potency of GamLPV in a randomized multicenter clinical trial on adult volunteers.

Materials and methods. In this multicenter, clinical, randomized, placebo-controlled, double-blind study 260 healthy adults aged 18–65 years were divided into 2 groups: G1 — 210 volunteers (GamLPV) and G2 — 50 volunteers (placebo). 0.25 ml GamLPV delivered to each nostril (5×10^9 CFU) 60 days apart. Levels of *Bordetella pertussis*-specific IgG, IgA antibodies in blood serum and levels of *B. pertussis*-specific secretory IgA antibodies in nasopharyngeal aspirates were measured by ELISA method and agglutination test. The dynamics of elimination of attenuated *B. pertussis* bacteria after the first and second intranasal administration of GamLPV to volunteers was estimated by using qPCR.

Results. Significant seroconversion of *B. pertussis*-specific IgG and IgA antibodies and growth of *B. pertussis*-specific secretory IgA antibody levels in nasal aspirates of volunteers were demonstrated. The dynamics of changes in the levels of IgG and IgA antibodies indicates a booster effect after second vaccination. Attenuated *B. pertussis* bacteria persist in the nose/oropharynx of vaccinated volunteers. The period of elimination after second vaccination is more than 2 times shorter than the period after the first one. The number of persistent *B. pertussis* bacteria after the second vaccination is less than 3% of the values after the first vaccination.

Conclusion. High immunogenicity and the formation of antibacterial protection after single and double intranasal vaccination of GamLPV have been proven.

Keywords: *vaccine GamLPV, Bordetella pertussis, multicenter clinical trial, volunteers, immunogenicity, protective potency, pertussis, humoral immune response*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Council of the Ministry of Health of the Russian Federation (extract No. 277 of 08.06.2021), the Ethics Committee of Infectious Diseases Hospital No. 1 (Moscow, meeting protocol No. 5 of 08.12.2021), the Ethics Committee of Eco-safety research center LLC LLC (extract No. 5 of meeting protocol No. 221 of 20.01.2022, extract No. 2 of meeting protocol No. 222 of 27.01.2022).

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ORIGINAL RESEARCHES

Оригинальное исследование
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Клиническое исследование иммуногенности и защитной активности живой рекомбинантной вакцины «ГамЖВК» интраназального применения для профилактики коклюша у взрослых добровольцев

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Аннотация

Введение. Рост заболеваемости коклюшем в мире, недостаточная продолжительность, эффективность и неспособность иммунитета, индуцированного бесклеточной коклюшно-дифтерийно-столбнячной вакциной, обеспечить противобактерийную защиту и предотвратить передачу возбудителя от человека к человеку обосновывают необходимость разработки новых противококлюшных вакцин. Живая коклюшная вакцина «ГамЖВК» интраназального применения прошла доклинические исследования в экспериментах с низшими приматами и две стадии клинических исследований на взрослых добровольцах, доказавшие её безопасность, иммуногенность и защитную активность. Отработаны метод и схема введения препарата.

Цель работы — подтверждение иммуногенности и защитной противобактерийной активности «ГамЖВК» в рандомизированном многоцентровом клиническом исследовании на взрослых добровольцах.

Материалы и методы. В многоцентровом клиническом рандомизированном плацебо-контролируемом двойном слепом исследовании приняли участие 260 здоровых добровольцев в возрасте 18–65 лет. Из них 210 человек были рандомизированы в группу вакцинированных «ГамЖВК» и 50 человек — в группу плацебо. Препарат «ГамЖВК» вводили дважды в каждый носовой ход по 0,25 мл (5×10^9 КОЕ) с интервалом 60 дней. Методом иммуноферментного анализа и реакции агглютинации определяли уровни специфичных к *Bordetella pertussis* IgG-, IgA-антител в сыворотке крови и IgA в назальных аспиратах. Методом полимеразной цепной реакции в реальном времени измеряли число геном-эквивалентов *B. pertussis* в ротоглоточных смывах для оценки динамики элиминации аттенуированных бактерий *B. pertussis* после первого и повторного введения «ГамЖВК» добровольцам.

Результаты. Показаны достоверные сероконверсия по специфическим IgG и IgA в крови и рост уровня секреторных IgA в назальных аспиратах добровольцев. Динамика изменения содержания IgG и IgA указывает на бустерный эффект после повторной вакцинации. Аттенуированные бактерии *B. pertussis* персистируют в носо- и ротоглотке вакцинированных. Время выведения бактерий после 2-й вакцинации сокращается более чем в 2 раза в сравнении с 1-м введением, при этом количество персистирующих бактерий после 2-й вакцинации составляет менее 3% от значений после 1-й вакцинации.

Заключение. Доказаны высокая иммуногенность и формирование противобактерийной защиты после однократной и двукратной интраназальной вакцинации добровольцев «ГамЖВК».

Ключевые слова: *вакцина «ГамЖВК», Bordetella pertussis, многоцентровое клиническое исследование, добровольцы, иммуногенность, защитная эффективность, коклюш, гуморальный иммунный ответ*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Советом по этике при Министерстве здравоохранения РФ (выписка № 277 от 08.06.2021), Этическим комитетом Инфекционной больницы № 1 (Москва, протокол заседания № 5 от 08.12.2021), Комитетом по вопросам этики при ООО «НИЦ Эко-безопасность» (выписка № 5 из протокола заседания № 221 от 20.01.2022, выписка № 2 из протокола заседания № 222 от 27.01.2022).

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Introduction

Pertussis is a highly contagious, airborne infectious disease controlled by vaccine prophylaxis. Vaccination against pertussis is included in the mandatory vaccination calendars of most countries of the world, starting from early childhood. According to the World Health Organization, 42 countries currently use tetanus-diphtheria-acellular pertussis vaccines (TDaP) containing an acellular pertussis component (aPC) and 143 countries use an adsorbed tetanus-diphtheria-pertussis vaccine. However, since the 2000s, the mass use of pertussis vaccine in economically developed countries has led to an increase in the incidence of pertussis in these countries, approaching in some years the level of the pre-vaccine period [1]. The age structure has changed towards an increase in the percentage of incidence among adolescents and adults [2]. The increasing incidence of pertussis among older children and adults has led to the realization of the necessity for revaccination. The feasibility of vaccination of pregnant women [3], mothers and close relatives to form a familial immunity to prevent infection of infants is being studied [4]. Low efficacy and short duration of immunity after aPC vaccination, as well as their inability to form antimicrobial immunity, are considered as one of the reasons for the increase in morbidity [5]. The reactogenicity of the pertussis corpuscular component and the low efficacy of aPC stimulate the development of new preparations capable of inducing long-term antibacterial immunity, which are not harmful during vaccination and are convenient for mass application.

The N.F. Gamaleya NRCEM has developed a live recombinant vaccine for intranasal administration for pertussis prophylaxis, called GamLPV. To date, the GamLPV vaccine has undergone preclinical studies and phase II clinical trials on adult healthy volunteers, which proved its safety, immunogenicity and protective activity, and the method and scheme of drug administration have been worked out [6–10]. A similar recombinant live pertussis vaccine (BPZE1) developed in France is at the stage of clinical trials [11–13].

The aim of this study is to confirm the immunogenicity and protective activity of the GamLPV vaccine when administered twice in adult volunteers aged 18–65 years in a multicenter clinical trial.

Materials and methods

Study design

A randomized placebo-controlled, blind, multicenter clinical trial was conducted to investigate the immunogenicity and safety of the GamLPV live vaccine according to:

- The Protocol of the clinical trial No. 03-GamLPV-2021, version 3 dated 28.01.2021, permission of the Ministry of Health of Russia No. 277 dated 08.06.2021.
- The Protocol of Clinical Trial No. 03-GamLPV-2021 was approved by the Ethics Council under the Ministry of Health of the Russian Federation (extract No. 277 dated 08.06.2021), as well as by local ethics committees:
- Ethical Committee of the Infectious Disease Clinical Hospital No. 1 of the Moscow Health Department (meeting protocol No. 5 of 08.12.2021);
- Ethics Committee of the Eco-safety research center LLC (extract No. 5 from meeting protocol No. 221 dated 20.01.2022; extract No. 2 from meeting protocol No. 222 dated 27.01.2022).

The inclusion and non-inclusion criteria relevant for assessing the immunogenicity of the vaccine used and the bacterial load in nasopharyngeal aspirates are summarized below. The full list of inclusion criteria, non-inclusion criteria, early exclusion of the volunteer from the study, composition and route of administration of the preparation, criteria for assessing the immunogenicity and efficacy of the preparation according to the Protocol are presented in the Appendix to the article on the journal's website.

When the protocol was being prepared, the level of IgG antibodies to *Bordetella pertussis* at the time of inclusion was used as one of the inclusion criteria in the study. The range of values of IgG antibody level was determined on the basis of data obtained using the Ridascreen test system. According to this system, volunteers with IgG antibody levels < 14 U/mL were considered seronegative (SN), antibody levels were considered indeterminate (gray zone, GZ) between 14 and < 18 U/mL, and volunteers with IgG antibody levels ≥ 18 U/mL were considered seropositive (SP). According to the protocol, both SN and mild to moderate SP volunteers with IgG antibody levels of 45 U/mL or less could be included in the study. By the time of inclusion of volunteers in the study, the Ridascreen test system was replaced by the ESR120G test system (Virion/Serion GmbH) due to organizational difficulties associated with the unavailability of the Ridascreen test system in Russia. According to the instructions for the ESR120G kit (Virion/Serion GmbH), samples with antibody levels < 40 IU/mL are considered to be SN, 40-50 IU/mL is considered to be the gray zone, and antibody levels

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of 50 IU/mL or higher are considered to be SP. An IgG antibody level of 45 IU/mL when determined using the Ridascreen kit is equivalent to an IgG antibody level of 126 IU/mL when determined using the ESR120G kit (Virion/Serion GmbH).

Inclusion criteria: men and women aged 18–65 years; medically stable condition, absence of pertussis-specific IgM antibodies, pertussis-specific IgG antibodies < 126 IU/mL (SERION ELISA classic ESR120G), absence of *B. pertussis* DNA in nasopharyngeal aspirates confirmed by polymerase chain reaction (PCR).

Exclusion criteria: history of pertussis; vaccination against pertussis within the last 10 years and any vaccination within the last year; any disease that, in the opinion of the investigator, may affect the results of the study or may lead to deterioration of health status during the study; reported severe post-vaccination complications in the history; taking medications for prophylactic or therapeutic purposes within 1 month before screening; participation in other clinical trials, presence of specific IgM antibodies to pertussis pathogen, specific IgG antibody level > 126 IU/mL (SERION ELISA classic ESR120G), presence of *B. pertussis* DNA in nasopharyngeal/ oropharyngeal aspirates.

Survey of volunteers and randomization

All volunteers participating in the study signed a voluntary informed consent form, after which they were assigned a number and screened. Compliance with all inclusion/non-inclusion criteria was confirmed before inclusion of volunteers in the study. The study population consisted of 260 male and female volunteers aged 18-65 years (inclusive), selected according to inclusion criteria and without non-inclusion criteria. Volunteers were allocated into 2 groups: those who received GamLPV (vaccinated group; n = 210) and those who received placebo (placebo group; n = 50) according to the protocol's randomization procedure. The vaccinated group was divided into 3 subgroups according to the content of specific pertussis IgG antibodies before the 1st vaccine administration: 1st subgroup, SN (n = 168); 2nd subgroup, GZ (n = 7); 3rd subgroup, SP (n = 35).

The clinical study included the 1st administration of the drug, observation period of 60 ± 5 days, and the 2nd administration of the drug and observation period of 60 ± 5 days. Determination of the level of specific IgG and IgA antibodies (enzyme-linked immunosorbent assay (ELISA), in blood serum, oro- and nasopharyngeal secretions) and titers of agglutination of bacterial suspension of pertussis pathogen (agglutination reaction, AR) by serum of volunteers at each drug administration were performed before administration (day 1), after administration on days 8, 15, 29 and 60. Determination of bacterial load in oro- and nasopharyngeal secretions by PCR at each drug administration was performed before drug administration, after the 1st administration after 1 h (day 1), on days 4, 8, 15, 29 and 60. Randomization and visit procedures are presented in the **Appendix** of the article on the journal website.

Study drug, doses and route of administration

GamLPV, a live vaccine for intranasal administration for the prevention of pertussis based on attenuated *B. pertussis 4MKS* bacteria [14], in the form of a lyophilizate for the preparation of a suspension for intranasal administration was produced by the Medgamal branch of the N.F. Gamaleya NRCEM. The vaccine series used in the clinical study passed all the necessary control stages.

The drug was administered at a dose of 5×10^9 CFU in the form of a suspension intranasally by injecting 0.25 mL into each nasal passage through a syringe without a needle, twice, with an interval of 2 months (60 ± 5 days). Sterile solution of stabilizer lyophilizate was used as placebo. Both preparations were reconstituted in 1 mL of 0.9% NaCl for injection.

Quantification of B. pertussis DNA

For real-time PCR (qPCR), we used DNA isolated from oropharyngeal and nasopharyngeal probes (hereinafter referred to as aspirates) placed in a single tube. After centrifugation, the samples were treated with guanidinedithiocyanate solution followed by DNA sorption on a sorbent [15]. To determine the amount of genome equivalent (GE) of *B. pertussis* DNA, the qPCR test system developed and validated by us was used [16]. qPCR was performed on the CFX-96 Touch thermocycler (Bio-Rad).

The elimination time of attenuated bacteria from the nasopharynx and oropharynx was estimated as a time interval in days from the moment of administration (1 h) until the established level was reached. The average *B. pertussis* DNA level of 0.7 GE/mL in both groups at the screening stage was taken as the level at which the elimination of bacteria was determined to be complete. If the indicated level was not reached, the elimination time was taken as 100 days. To compare the groups of volunteers after the 2nd bacterial administration and the 1st vaccination, we used the Kaplan–Meier method (Survival Analysis — Comparison of Two Samples module of the Statistica v. 10.0 program), which allows the use of censored data. Cases that did not reach the set level of 0.7 GE/mL were classified as censored data. Such cases were reported after the 1st vaccination, representing 6.2% (13 of 210), and 1 case less than 1.0% after the 2nd vaccination (1 of 202). The appropriateness of this method is because, as with the survival analysis, not all individuals had completed excretion by the time the study was completed (censored data), but they were not excluded but were used in the analysis. Differences were assessed using the log-rank test.

Methods for assessing the immunogenicity of GamLPV

Immunogenicity of GamLPV was evaluated by induction of specific IgG and IgA antibodies in the blood serum of volunteers detected by ELISA and AR. Local immune response was evaluated by the dynamics of specific secretory IgA antibodies to *B. pertussis* in nasopharyngeal aspirates.

Blood samples were collected in vacuum tubes with blood clotting activator (Vacuette). Serum was collected after centrifugation of samples for 20 min at 300g to thicken the clot. The level of specific IgG and IgA antibodies to pertussis pathogen in serum was determined by ELISA according to the instructions of the manufacturer of the ESR120G, ESR120A and ESR120M test systems (Virion/Serion GmbH). IgA antibodies in aspirates, as well as in blood serum, was determined using the ESR120A test system (Virion/Serion GmbH), but the samples were diluted 2-fold instead of 100 (according to the manufacturer's instructions). To obtain absolute values of antibody concentration in aspirates, the values calculated from the calibration curve were divided by 50.

AR was performed on kits produced by Research and Production Association Ecolab. The titer was considered to be the highest serum dilution at which an AR of at least "+++" (three crosses) was obtained.

Statistical processing of data

Mathematical and statistical analysis of the results was performed using the Statistica v. 10.0, R 4.2.1 and Microsoft Office Excel 2013 software packages to generate graphs and tables.

The obtained quantitative data were checked for compliance with the law of normal distribution. To determine normality, the Kolmogorov–Smirnov criterion (for a sample size of more than 50 volunteers) or the W Shapiro–Wilk criterion (for a sample size of less than 50 volunteers) were used. If the statistic was significant, the hypothesis of normal distribution of the variable values was rejected.

Immunogenicity in the studied groups represented by qualitative features (fractions) was assessed using the χ^2 test criterion when the expected frequency of occurrence of a feature was more than 5 or the two-sided Fisher's exact test when the condition for the χ^2 criterion was not met.

Immunogenicity points for qualitative criteria are presented as absolute frequencies (trait occurrence), relative frequencies (%), and 95% confidence interval of the proportion (Clopper-Pearson).

Quantitative criteria of immunogenicity were evaluated using nonparametric methods (Mann–Whitney test) or Student's t-test for independent samples according to the nature of distribution. The data were also presented taking into account the nature of distribution.

To compare several independent samples (more than 2) represented by quantitative variables, we used the Kraskell–Wallis rank analysis of variances. If statistically significant differences between all groups were established, the "a posteriori" method "Comparison of mean ranks for all groups" (nonparametric Dunn's test), which takes into account the problem of multiple comparisons, was used to identify differences between individual groups.

In addition to the descriptive statistics given for safety, geometric mean values with 95% confidence intervals were used to represent agglutination titers.

Log-rank test was used to compare groups of volunteers after the 1st and 2nd bacterial administration.

Two-sided criteria were used for immunogenicity points.

The magnitude of the error to confirm the null hypothesis should be greater than 0.05 (at $p \leq 0.05$ the null hypothesis is rejected, at $p > 0.05$ — accepted).

Results

Dynamics of specific anti-pertussis IgG and IgA antibodies in serum and IgA antibodies in nasopharyngeal and oropharyngeal aspirates.

The results of IgG and IgA antibody measurements by subgroups are presented in **Figs. 1, 2**. The significance of statistical differences between the total vaccinated group and placebo was assessed using the Mann–Whitney test. Statistically significant differences between subgroups were assessed using nonparametric analysis of variance — Kraskell–Wallis rank analysis of variances and Dunn's posterior nonparametric test (tables not shown).

There were no significant differences in IgG antibodies between the total vaccinated group and the placebo group at the time of administration of GamLPV. On the 8th day after the 1st administration, IgG antibody levels in the total vaccinated group increased significantly compared to the placebo group ($p = 0.012$); starting on the 15th day after the 1st vaccination, differences between groups became highly statistically significant ($p < 0.0001$). Compared to baseline (19 IU/mL), median IgG antibody levels in the vaccinated group after the 1st administration of the drug on days 8, 15, 29, and 60 increased 1.1, 2.3, 3.6, and 3.8-fold, respectively. After the 2nd administration of GamLPV, the multiplicity of increase in IgG levels on days 8, 15, 29, and 60 was 3.7, 4.2, 3.9 and 3.8-fold, respectively (70.5–79.5 IU/mL). In the placebo group, median IgG antibody levels did not change significantly and ranged from 16.8–21.0 IU/mL during the course of the study.

Before the 1st administration of GamLPV, the median IgG level in each subgroup (SN, GZ, SP) was significantly different from the median of the total vaccinated group. Significant differences were also observed between the subgroups of SN and SP, SN and GZ. Within the observation period, the median IgG antibody level in the SP subgroup was significantly different from the

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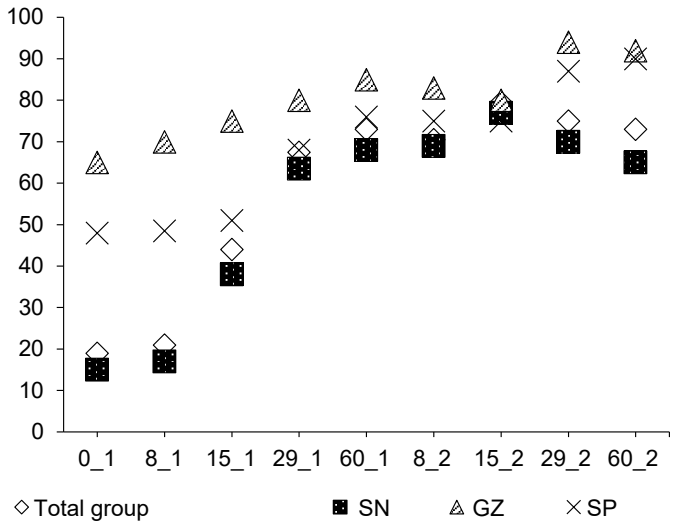

Fig. 1. *B. pertussis*-spesific IgG antibody dynamics in blood serum of vaccinated volunteers and additional groups SN, GZ and SP.

Here and on the Figs. 2–4: days after administration of GamLPV to volunteers from additional groups are plot on X-axis: 0_1 — the 1st day of administration of GamLPV (before administration), 8_1, 15_1, 29_1, 60_1 – 8, 15, 29 and 60 days after the 1st administration of GamLPV respectively; 8_2, 15_2, 29_2, 60_2 — 8, 15, 29, 60 days after the 2nd administration of GamLPV respectively. IgG antibody concentration (IU/mL) in the blood serum of volunteers is plot on Y-axis.

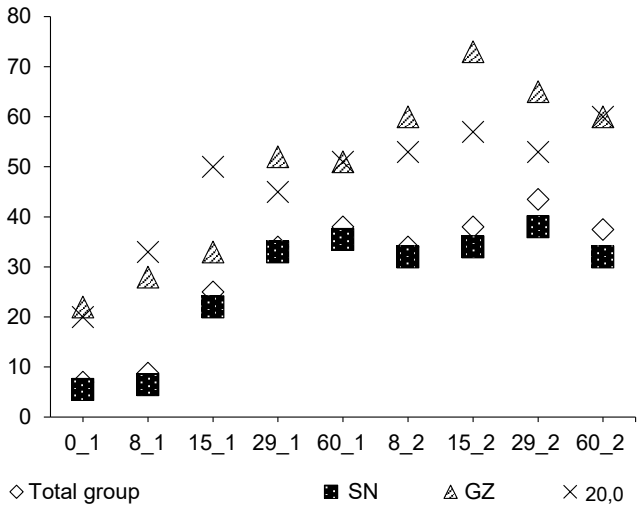

Fig. 2. *B. pertussis*-spesific IgA antibody dynamics in blood serum of vaccinated volunteers and additional groups SN, GZ and SP.

IgA antibody concentration (IU/mL) in the blood serum of volunteers is plot on Y-axis.

median for the total vaccinated group on day 15 after the 1st administration ($p = 0.001$), and on days 29 ($p = 0.023$) and 60 ($p = 0.001$) after the 2nd administration of GamLPV. Significant differences between the SN and SP groups were observed on days 15 ($p = 0.001$) and 29 ($p = 0.023$) after the 1st administration and on days 29 ($p = 0.003$) and 60 ($p = 0.001$) after the 2nd administration of GamLPV. IgG antibody levels in the GZ subgroup were not significantly different from values for the other subgroups or the total vaccinated group at any of the post-vaccination controls.

When assessing the level of specific IgA antibodies in the blood, significant differences were found between the vaccinated and placebo groups — 7.0 and 4.8 IU/mL, respectively ($p = 0.014$). Both values were significantly less than the seronegativity cut-off limit (< 25 IU/mL). After the 1st vaccination from day 8 onwards, the significance of differences between groups became highly significant ($p < 0.0001$) and remained at this level until the end of the study. In the vaccinated group after the 1st administration, the median values on days 8, 15, 29 and 60 increased 1.3, 3.6, 4.9 and 5.4 times, respectively, compared to baseline. After the 2nd vaccination, a booster effect was observed on day 29 — IgA antibody levels increased 6.2-fold compared to baseline. By day 60, the IgA level returned to the level detected before the 2nd administration. In the placebo group no significant changes were found during the observation period — the median values ranged from 4.0–6.0 IU/mL. Subgroup analysis showed that at the time before the 1st administration of GamLPV the level of IgA antibodies to *B. pertussis* significantly differed between the SP and SN subgroups ($p = 0.001$), while in the SP subgroup the antibody level also significantly differed from the value characteristic of the general group of vaccinated persons ($p = 0.001$). At subsequent follow-up checkpoints, up to day 8 after the 2nd administration of GamLPV, there were no significant differences between the subgroups. On day 15 after the 2nd administration, significant differences were again found between the SP and SN subgroups ($p = 0.001$), as well as between the SP subgroup and the value for the total vaccinated group ($p = 0.001$). On days 29 and 60 after the 2nd administration, significant differences in IgA antibody levels to *B. pertussis* persisted only between the SP and SN groups ($p = 0.049$). For the SP subgroup, no significant differences in the median IgA antibody level with the other subgroups were found at any of the study controls.

Determination of specific secretory anti-pertussis IgA antibodies in nasopharyngeal and oropharyngeal flushes from volunteers revealed statistically highly significant ($p < 0.0001$) differences between the vaccinated and placebo groups at all controls (**Fig. 3**). In the vaccinated group, compared to baseline (0.20 IU/mL), the median levels of secretory IgA antibodies on days 15, 29 and 60 after the 1st vaccination increased 2.5, 4.5 and 3.2-fold, respectively. This increased continuously after the 2nd vaccination and on days 8, 15 and 29, the multiplicity of increase was 4.4, 5.2 and 5.8, respectively. By day 60, the median level of secretory IgA antibodies returned to the values detected on day 60 after the 1st administration of GamLPV. Subgroup analysis showed significant differences in secretory IgA antibody levels between the SN and SP subgroups on day 29 after the 2nd vaccine administration ($p = 0.031$).

Dynamics of change in the proportion of volunteers with more than twofold increase in the level of specific anti-pertussis IgA and IgG antibodies in serum and nasopharyngeal aspirates. The multiplicity of increase in the amount of specific anti-pertussis IgA and IgG antibodies in aspirates and in serum at each visit was analyzed in comparison with the data before drug administration (**Fig. 4**).

The proportion of volunteers with at least a twofold increase in the level of IgG antibodies in the blood in the vaccinated group was significantly different (χ^2 test, $p < 0.0001$) from the placebo group as early as day 15 after the 1st injection, and the differences remained significant until the end of the study. On days 15, 29, and 60 after the 1st administration and on days 8, 15, 29 and 60 after the 2nd administration, the proportion of volunteers who achieved this rate was 46.4, 69.3, 72.8, 73.1, 75.0, 77.9, and 77.0%, respectively. Subgroup analysis showed that in the SN subgroup, at day 29 after the 1st administration, the proportion was 84.3%. This value increased at the following time points, reaching a maximum of 92.4% on day 29 after the 2nd administration. In the SP and GZ subgroups, these values ranged from 11.4–17.1 and 14.3–57.1% during the same period, respectively.

When assessing IgA antibody levels, it was found that from day 15 after the 1st injection until the end of the analysis, the proportion of patients with at least a twofold increase in serum IgA antibody levels in the vaccinated group differed from the placebo group with high reliability (χ^2 test, $p < 0.0001$). On days 15, 29 and 60 after the 1st administration, the percentage of such volunteers in the vaccinated group was 71.8, 85.1 and 86.1%, respectively. After the 2nd vaccination on days 8, 15, 29 and 60, this proportion in the vaccinated group was 88.1, 88.1, 90.0, and 89.0%, respectively. Subanalysis of subgroups showed that in the SN subgroup, a twofold increase in the level of IgA antibodies in blood was observed in more than 90.0% of volunteers from day 29 of the study to the end of the study (91.6–94.9%). In the SP subgroup these values ranged from 60.0–74.3%, in the SP subgroup — 57.1–85.7%.

The dynamics of the level of secretory IgA antibodies was similar to that of IgA antibodies in serum. Highly significant differences ($p < 0.0001$) between the vaccinated and placebo groups were detected from day 15 after the 1st vaccine injection. The proportion of volunteers with a twofold increase in IgA antibody levels on days 15, 29 and 60 after the 1st administration and on days 8, 15, 29 and 60 after the 2nd vaccine administration was 58.4, 71.6, 70.8, 80.2, 82.6, 84.0 and 73.5%, respectively. There were no significant differences between the SN, GZ and SP subgroups, except for one point, 29 days after the 2nd vaccine administration, at which the SN and SP subgroups differed significantly ($p = 0.031$).

Timing of reaching maximum values of IgA and IgG antibodies in serum after the 2nd vaccination compared to the 1st vaccination. In order to analyze the time to reach the maximum concentrations, the data of only those volunteers of the vaccinated group were analyzed, in which the antibody level was evaluated at least in 3 points, except for the time of drug administration.

After the 2nd vaccination, a significant reduction in the time to reach maximum antibody levels was observed: more than two-fold for serum IgG and 4-fold for serum IgA; two-fold for secretory IgA (**Table**).

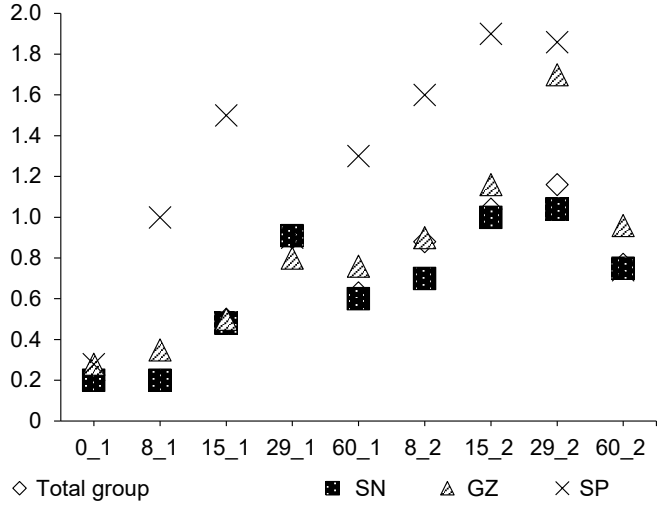

Fig. 3. *B. pertussis*-specific secretory IgA antibody (median values) dynamics in nasopharyngeal/oropharyngeal aspirates of vaccinated volunteers and additional groups SN, GZ and SP.

Secretory IgA antibody concentration (IU/ml) in nasopharyngeal/oropharyngeal aspirates is plot on Y-axis.

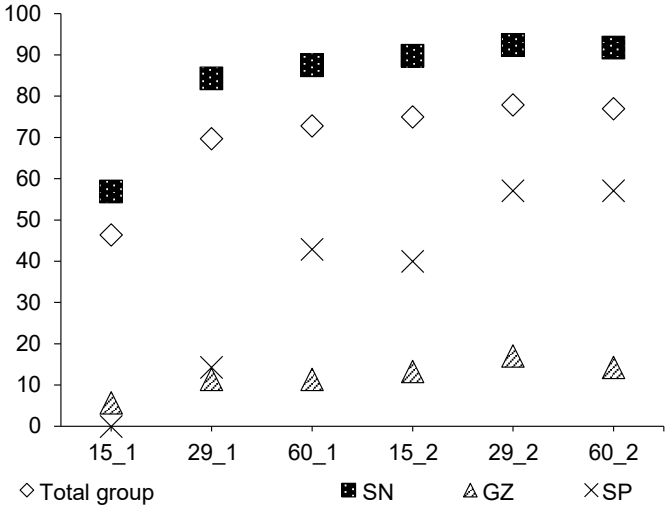

Fig. 4. The dynamics of the proportion of vaccinated volunteers with more than a twofold increase of *B. pertussis*-specific IgG antibodies in blood serum.

The proportion of volunteers (%) with more than a twofold increase of *B. pertussis*-specific IgG antibodies in blood serum is plot on Y-axis.

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Comparative evaluation of *B. pertussis*-specific IgA and IgG antibody median values in blood serum and secretory IgA antibodies in nasopharyngeal/oropharyngeal aspirates of volunteers with time to Cmax in group of vaccinated volunteers after the 1st and the 2nd vaccination

Class of antibodies	Vaccination number	*n*	Median value of specific antibody content, IU/mL	p_1	Median value of bacterial elimination time, day	p_2
Serum IgA	1	207	44,0	0,006	56,0	< 0,0001
	2	200	55,5		14,0	
Serum IgG	1	208	77,5	< 0,0001	59,0	< 0,0001
	2	200	90,0		28,0	
Secretory IgA	1	208	1,98	< 0,0001	28,0	< 0,0001
	2	199	6,28		14,0	

Note. Reliability of differences was calculated using the Wilcoxon test.

Evaluation of the immune response to vaccination of GamLPV volunteers using AR. The immune response, which characterizes the level of agglutinating antibodies in the serum of vaccinated volunteers, was assessed by the AR method. Antibody titers were presented as geometric mean values with 95% confidence intervals and descriptive statistics for asymmetrically distributed data (tables not presented).

Statistically significant differences between the groups of vaccinated volunteers and those who received placebo were established at all points analyzed. Starting on day 15 after the 1st vaccination, these differences became statistically highly significant ($p < 0.0001$). The antibody titer in the placebo group did not change significantly throughout the entire examination period.

In the vaccinated group, the geometric mean agglutination titer on day 8 after the 1st vaccination (45.9) was generally consistent with the baseline (42.4). An increase in titer was observed from day 15 after the 1st vaccine administration. The multiplicity of increase in the geometric mean agglutination titer on days 15, 29 and 60 was 1.7, 2.3, and 2.0, respectively. After the 2nd vaccination on days 8, 15 and 29, the multiples were 2.0, 2.6 and 2.7, respectively. By day 60 after the 2nd vaccination, the titer decreased slightly, but the multiplicity increase of 2.6 exceeded that on day 60 after the 1st vaccination.

The proportion of volunteers with an agglutination titer of 1 : 80 or higher on days 8, 15, 29, and 60 after the 1st vaccination was 38.1, 56.0, 73.6, and 65.8%, and 66.8, 77.6, 77.6 and 80.0% after the 2nd vaccination. After the 1st vaccination, the proportion of volunteers with an agglutination titer of 1 : 80 in the total vaccinated group was statistically significantly different from the distinguished subgroups. After the 2nd vaccination, no statistically significant differences were found between subgroups. The highest percentage of individuals who met the specified criterion was found in the group of SP volunteers (from 80.0% on day 15 after the 1st administration to 94.3% on days 15 and 60 after the 2nd vaccine administration). In the SN subgroup, for the same time interval, this indicator ranged from 51.5–75.9%, in the SP subgroup — 42.9–100.0%.

Elimination time of attenuated bacteria from the nasopharynx after the 2nd administration of bacteria compared with the 1st vaccination. **Fig. 5** shows the dynamics of elimination of attenuated bacteria from the nasopharynx as a decrease in the cumulative proportion of persons with non-eliminated *B. pertussis* bacteria in separate time intervals. From the presented data it is evident that elimination of vaccine *B. pertussis* from the nasopharynx after the 2nd administration of bacteria is faster than after the 1st vaccination of volunteers. At the same time, after the 2nd vaccine administration, there is a greater proportion of volunteers whose elimination process was completed early — within the first 7 days after vaccination.

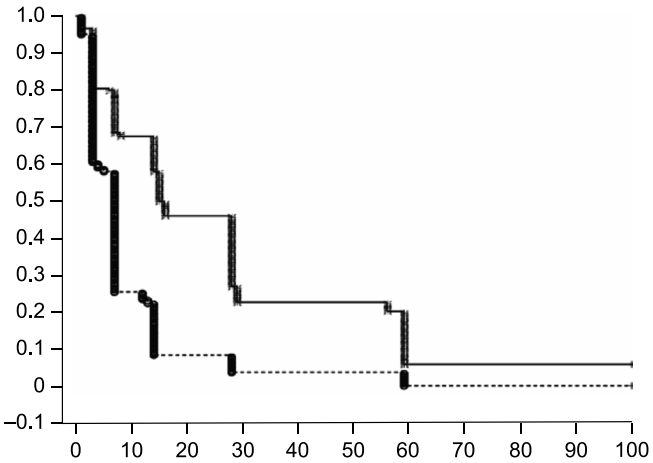

Fig. 5. The dynamics of cumulative fraction of volunteers who did not eliminate *B. pertussis* bacteria from the nasopharynx after second administration of bacteria compared with the first vaccination.

Time duration of *B. pertussis* bacteria elimination starting from the first hour after GamLPV administration are plot on X-axis; the cumulative fraction of volunteers in whose aspirates the DNA of *B. pertussis* bacteria was detected by PCR in an amount more than 0,7 GE/mL, is plot on Y-axis. The dashed line indicates the second vaccination schedule of volunteers.

Comparative evaluation of the elimination time of attenuated bacteria from the nasopharynx of vaccinated volunteers of the general group after the 1st and 2nd vaccinations using the log-rank criterion showed a statistically highly significant ($p < 0.0001$) decrease in the elimination duration after the 2nd vaccination from 16 to 7 days according to the median value. The SN subgroup showed the greatest difference in the duration of bacterial elimination period after the 1st and 2nd vaccinations, 28 and 7 days, respectively. In the SP subgroup, the differences were also significant ($p = 0.013$) — the median duration of bacterial elimination was 14 and 7 days, respectively.

In addition to the elimination time analysis, the total amount of *B. pertussis* DNA in the nasopharynx of volunteers, during follow-up after the 1st and 2nd vaccinations, was estimated using the linear trapezoidal method. The area under the curve after the 1st vaccination was 6714 (1539) GE day/mL and after the 2nd vaccination was 443 (44) GE day/mL at the median when calculated between days 3 and 60.

Discussion

The results of studying the immunogenicity of GamLPV and virulent *B. pertussis* bacteria on the experimental model of nonhuman monkeys showed that the first contact with infection induced a slow and unexpressed growth of specific IgG antibodies, lasting, as a rule, until the 46th–60th day of observation. Repeated administration of bacteria resulted in a rapid and pronounced increase in IgG antibodies starting on day 3–7 with a maximum on day 14–28 after the 2nd infection [6, 7]. The results of the phase I clinical trial GamLPV confirmed the slow increase of IgG antibody values after the 1st vaccination [8]. At that time, it was also shown that, at least among volunteers in Moscow and the Moscow region when using the Ridacreen kit, less than 50% of adults aged 40–60 years were SN [8–10].

The tested vaccine is intended for revaccination of adults regardless of their health status and level of specific IgG antibodies. Therefore, it is of practical interest to study the tolerability, immunogenicity and protective activity of GamLPV for volunteers 18–65 years of age who have no obvious health disorder, no symptoms of pertussis and serum IgG antibody level lower than the threshold defined in the country for laboratory diagnosis of pertussis. The lack of studies in Russia determining the serologic status of the population (IgG, IgM and IgA antibody levels) and the absence of in-house ELISA test systems does not allow quantitative assessment of the level of protective antibodies after vaccination and/or disease. Therefore, based on the results of our previous studies and some data from the national literature, an IgG antibody value of 45 U/mL [9, 10], measured using the Ridacreen kit, was accepted as the threshold for inclusion in the study, which allowed more than 80% of screened volunteers to be included in the study. The impossibility of using Ridacreen test systems in the study presented in this article led to the necessity to determine the conversion factor for converting the value of the IgG inclusion criterion limit point < 45 U/mL into international units (IU/mL) measured using the available and registered in Russia ESR120G test system (Institut Virion/Serion GmbH). As a result of semi-empirical calculations, a value equal to 2.8 was accepted as such a coefficient. Thus, the level of 126 IU/mL determined by the ESR120G test system (Virion/Serion GmbH) was taken as the upper limit of the IgG antibody level at which a volunteer could be included in the study. The calculated coefficient was used only to determine the boundary in the inclusion criteria. All other values of IgG and IgA antibody parameters of blood serum of volunteers and aspirates were determined using the ESR120A and ESR120G test systems (Institut Virion/Serion GmbH) in the units used by them (IU/mL).

The choice of parameters and criteria (control points) for assessing the immunogenicity of GamLPV intranasal application by the main indicators is traditional for multicenter studies and is justified in protocols, brochures and reports on the conducted clinical trials. Control points characterizing the level of immunogenicity of the drug are given in the Appendix to the article on the journal's website.

The conducted study demonstrated a significant seroconversion of IgG and IgA antibodies and an increase in the level of secretory IgA antibodies in nasopharyngeal aspirates of volunteers. The values obtained exceeded the baseline antibody levels in volunteers included in the vaccinated group and the antibody levels in volunteers in the placebo group. Significant differences between the levels of IgG and IgA antibodies in the vaccinated and placebo groups were observed already on day 8. Thus, the humoral immune response to intranasal vaccination of volunteers with GamLPV begins to manifest itself one week after the drug administration. The maximum increase of median values of antibody levels to *B. pertussis* in the serum of volunteers was 6.2 times for IgG and 4.2 times for IgA. The level of IgA antibodies in aspirates maximally increased 5.8 times after the 2nd vaccination. Similar dynamics was registered when assessing seroconversion by AR indicator.

The time to reach maximum levels of IgG and IgA antibodies in serum and secretory IgA antibodies in nasal aspirates after the 1st and 2nd vaccination of volunteers is indicative. In all cases, antibody levels, regardless of isotype, not only exceeded those after the 2nd administration but also peaked at shorter times after the 1st administration.

An important indicator is the proportion of volunteers who reached the target value of exceeding the initial antibody level. The control point chosen by us and included in the protocol assumes achievement of

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at least a twofold increase in IgG and IgA antibody levels in at least 80% of vaccinated volunteers. When calculated for the total group, this reference point was achieved and exceeded for IgA antibody levels in serum and in aspirates. For IgG antibodies, a twofold increase in antibody levels was observed in 77.9% of vaccinated volunteers.

Analysis of the dynamics of changes in the level of IgG and IgA antibodies in serum by subgroups confirmed the general dynamics of increasing levels of IgA and IgG antibodies. Quite expectedly, the multiplicity of increase in the total vaccinated group and the SN subgroup were close to each other and significantly exceeded the multiplicity of increase in the SP and GZ subgroups (Fig. 4), which is due to the effect of the base (initial antibody level against which the multiplicity was calculated). The insignificant lag in the proportion of volunteers who achieved a twofold increase in the level of IgG antibodies from 80% in the total group of the vaccinated is fully explained by the presence in it of volunteers of the SP and GZ subgroups with initially high IgG antibody values and, accordingly, the impossibility of achieving a high multiplicity of antibody increase by them. Thus, on the 29th day after the 2nd vaccination, the proportion of volunteers with a twofold or more increase in the level of IgG antibodies in serum among all vaccinated persons was 77.9%, whereas in the SN subgroup this level exceeded 92%, in the SP subgroup it was only 17.1%, and in the GZ subgroup — 57.1%. The SP and GZ subgroups together account for about 20% of the total vaccinated group, which affected the final value and determined the minimum value (2.1%) of this indicator, which was insufficient to achieve the stated primary endpoint of 80.0% of patients with a twofold increase in IgG antibody levels.

It should be noted that our chosen level of 80% and the achievement of this level with double seroconversion were determined by the results of the previous stage of the study, which included only SN by IgG antibody volunteers. As can be seen from the data obtained, the reported rate was achieved in the SN group [8–10]. Moreover, in similar experiments conducted during clinical trials of the BPZE1 vaccine, the authors calculated the proportion of patients with a 1.5-fold increase in antibody levels and reached the control point only at this criterion [13]. Recalculation of our values for the 1.5-fold increase showed achievement of this parameter in our study in more than 80% of volunteers starting from day 29 after the 1st administration of GamLPV.

Assessment of immunogenicity of GamLPV using AR did not reveal significant differences with the results obtained by ELISA method, except for a more pronounced increase in the proportion of volunteers with agglutination titer 1 : 80 and higher in the SP subgroup compared to SN, while the dynamics of IgA and IgG antibody levels in these groups is of the opposite nature. Reliability and biological meaning of the revealed differences require further investigation. Generalization of the results of the analysis of blood serum of volunteers in two clinical trials of the candidate GamLPV vaccine, as well as the data obtained in the study of monkeys immunized with GamLPV and/or experimentally infected with virulent bacteria of the pertussis pathogen, showed that ELISA and AR in general reveal the general picture of immunological reactions of the organism to immunization and experimental infection. The use of AR in most cases did not reveal significant differences from the results obtained by ELISA, and the differences in AR results obtained using different series of the preparation, with good reproducibility of ELISA results, indicate low standardization of the kits used for staging AR. The obtained results make it inexpedient to use AR in further clinical studies of GamLPV. The non-alternativity of ELISA to determine the serologic status of patients and vaccinated people once again emphasizes the necessity to develop and produce a domestic ELISA test system for quantitation of antibodies to the main antigens of the pertussis pathogen.

There were no significant differences in the level of secretory IgA antibodies in aspirates, whereas the levels of IgA and IgG antibodies in blood serum differed significantly between subgroups, including before the beginning of the experiment, which is especially pronounced when comparing the subgroups of SN and SP. The observed differences in median values of IgA- antibody levels in serum before vaccination are apparently due to the correlation between IgG and IgA antibody levels in volunteers and reflect the effects of prior exposure to *B. pertussis* antigens.

The dynamics of the proportion of volunteers with more than twofold increase of IgA antibodies in aspirates did not differ significantly in the subgroups and reached the control values of 80% on day 15–29 after the 2nd vaccination. The noted feature may indicate the relative independence of local immunity formation from the initial IgG antibody level. The second vaccination enhances the local response irrespective of the serologic status of the volunteer, which once again indicates the extremely important role of local immune defense in the formation of antibacterial immunity in pertussis. This is also indicated by the results of determining the bacterial load in the naso- and oropharynx of vaccinated volunteers in dynamics. For each of the subgroups, with the exception of GZ, a sharp decrease in the time of bacterial elimination after the 2nd vaccination and the absence of a pronounced difference in the value of parameters for different subgroups of volunteers were noted.

The number of bacteria registered in volunteers during the observation period after the 1st and 2nd immunizations can be considered as an additional value to the bacterial elimination time in relation to the assessment of the antibacterial activity of GamLPV. This value was estimated by calculating the area under the bacterial

elimination curve after each immunization. Analysis of the presented data indicates that the elimination efficiency for the period from day 3 to day 60 after the 2nd vaccination was 33 times higher compared to the 1st vaccine administration. Since it takes time to develop an immune response, it is most appropriate to determine the area under the curve not from day 1, when the body has not yet had time to react to the introduction of bacteria, but from day 3 to day 60. In addition, the results of measuring IgG and IgA antibody values indicate that the immune response of the organism after the 1st and 2nd vaccinations is manifested closer to the 8th day after the introduction of bacteria.

The above results of the analysis of the elimination efficiency of attenuated bacteria after the 2nd vaccine administration suggest that the level of specific IgG and IgA antibodies in serum does not fully reflect the level of antibacterial protective activity. This is consistent with the statement that there are no correlations between serum IgG antibody levels and the protective activity of pertussis vaccines in practice [5]. This statement, at first glance, limits the use of serum IgG and IgA antibody levels in pertussis vaccine immunogenicity studies. Seroconversion is undoubtedly an important characteristic of the humoral immune response in pertussis or vaccination, and the antibodies produced participate in the formation of immune protection aimed at neutralizing the toxic activity of the pathogen and preventing clinical manifestations of the disease, but it does not indicate the formation of sterile antibacterial immunity, as after reconvalescence or vaccination with live pertussis vaccine. It is now generally accepted that the cellular component of immunity is responsible for the formation of antimicrobial defense in pertussis disease. Direct experiments on monkeys demonstrate that the presence of all isotypes of specific antibodies to pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae after their immunization with cell-free pertussis vaccine does not ensure prevention or at least inhibition of virulent bacteria multiplication in the nasopharynx after experimental infection of animals [5]. The demonstrated absence of dependence of bacterial elimination time on the initial level of IgG and IgA antibodies in blood serum before vaccination can be considered as an additional argument in favor of the made assumption. To assess the induced antibacterial response, most likely, data on the level of IgA antibodies in oro- and nasopharyngeal washes and cellular response parameters, primarily characterizing the Th17 response, should be used.

The recently published results of the BPZE1 vaccine clinical trial are generally consistent with those presented in the present study [13]. Furthermore, they contain a comparison of BPZE1-induced and TDaP-induced immune response rates. According to the authors, the level of seroconversion after intranasal vaccination of BPZE1 volunteers is slightly lower, but comparable to the values of IgG and IgA antibodies in the serum of volunteers after TDaP vaccination. At the same time, injectable TDaP vaccination, unlike intranasal BPZE1, is not able to induce specific secretory antibodies in the nasopharynx of volunteers. The high level of tolerability of the BPZE1 vaccine and the safety of its intranasal administration in adult volunteers have been demonstrated.

Conclusion

The results of this study showed immunologic efficacy (immunogenicity) of the GamLPV live pertussis vaccine when administered twice and once intranasally in comparison with placebo in adult volunteers. The basis was the achievement of immunogenicity control points of the vaccine:

- The proportion of volunteers with more than two-fold increase of IgG and IgA antibody levels in serum after vaccination was more than 80%;
- The proportion of volunteers with an agglutination titer of 1 : 80 in AR after vaccination was more than 80%;
- The proportion of volunteers with more than two-fold increase of IgA antibody levels in naso- and oropharyngeal aspirates after vaccination was more than 80%.

A statistically significant decrease in the time to reach maximum values of IgA antibodies in blood serum and naso- and oropharyngeal aspirates after the 2nd vaccination in comparison with the 1st and a reduction in the elimination time of attenuated bacteria from the nasopharynx after the 2nd administration of bacteria in comparison with the 1st vaccination of volunteers, assessed by the number of GE (using qPCR) were shown.

The difference in the proportion of volunteers with a more than twofold increase in IgG antibody levels in SN volunteers (more than 90%) and in SP volunteers (less than 78%) is due to the initially high antibody levels of the latter and, consequently, the lack of doubling after the next exposure to infection. The increase in the proportion of volunteers with more than doubling of IgA and IgG antibody levels is mainly due to SN volunteers with lower initial IgA antibody levels.

The contribution of SN and SP volunteers to the proportion of volunteers with more than a twofold increase in secretory IgA antibodies (> 80%) is approximately equal, with close initial median IgA antibody values in each group, but larger values achieved in SP-volunteers. The latter result may indicate a short period of secretory IgA antibody presence after infection/vaccination, but accelerated production and attainment of higher values in SP volunteers with previous contact with infection.

The reduction of bacterial elimination time after the 2nd vaccination compared to the 1st is equally recorded in SN and SP volunteers and is 2.0 and 2.3 times in each group, respectively.

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Summarizing the presented results, it can be stated that the 1st intranasal vaccination of volunteers leads to the formation of specific humoral response of the organism, including the production of specific secretory IgA antibodies. Repeated administration of attenuated bacteria enhances the immune response and demonstrates the presence of antibacterial immunity formed as a result of the 1st intranasal vaccination of volunteers with the preparation GamLPV. A single intranasal administration of the preparation may be sufficient for revaccination of the adult population with GamLPV. The necessity for double vaccination of young children and adolescents should be determined by the results of clinical trials.

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Selective suppression of influenza A/H5N1 virus replication *in vitro* using nanocomplexes consisting of siRNA and aminopropylsilanol nanoparticles

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Abstract

Relevance. Studies on model systems have confirmed the effectiveness of antisense oligonucleotides, including those that contain photoactive groups, for the modification of nucleic acids. However, this strategy has not yet found wide application due to the lack of successful methods for the intracellular delivery. The development of effective preparations capable of acting on target nucleic acids in cells is an urgent task.

The aim of the study is to create nanocomplexes consisting of aminopropylsilanol nanoparticles and short interfering RNA (siRNA) to study their effect on target nucleic acids by the example of inhibition of influenza A virus replication in vitro.

Materials and methods. MDCK cells, influenza virus A/chicken/Kurgan/05/2005 (A/H5N1), aminopropylsilanol nanoparticles, and native and modified siRNA molecules.

Results and discussion. We have prepared unique Si~NH_2/siRNA nanocomplexes, which consist of aminopropylsilanol nanoparticles and siRNA molecules, which enable cell penetration and selective interaction with target nucleic acids, respectively. The antiviral activity of the proposed nanocomplexes has been studied on MDCK cells infected with the influenza A/H5N1 virus. It has been shown that the double-stranded siRNA molecules in the nanocomplexes, which act by the RNA interference mechanism, are more efficient in inhibiting the replication of the influenza virus than the corresponding single-stranded RNA fragments. The most effective nanocomplex that contained siRNA targeted at the chosen region of mRNA segment 5 of the viral genome reduced virus replication in the culture by a factor of 630. We have shown that non-agglomerated and water-soluble aminopropylsilanol nanoparticles are low-toxic, capable of delivering siRNA into cells and protecting siRNA in the Si~NH_2/siRNA nanocomplexes from hydrolysis by cellular nucleases.

Conclusion. The biological activity of the created nanocomplexes has been demonstrated by the example of highly effective selective suppression of influenza A/chicken/Kurgan/05/2005 virus replication in the cellular system.

Keywords: *aminopropylsilanol nanoparticles, nanocomplexes, siRNA, antiviral activity, influenza A/H5N1 virus*

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Оригинальное исследование
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Селективное подавление репликации вируса гриппа A/H5N1 *in vitro* с помощью нанокомплексов, состоящих из siRNA и наночастиц аминопропилсиланола

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Аннотация

Актуальность. Вирусы гриппа, относящиеся к семейству *Orthomyxoviridae*, широко распространены в природе и часто являются причиной возникновения пандемий. Появление новых штаммов вируса, устойчивых к лекарственным препаратам, вызывает потребность в разработке новых эффективных лекарственных форм, селективно действующих на вирусы гриппа А.

Цель работы — создание нанокомплексов, состоящих из наночастиц аминопропилсиланола (АПС) и малых интерферирующих РНК (siRNA), и исследование их воздействия на нуклеиновые кислоты-мишени на примере ингибирования репликации вируса гриппа А в клеточной системе.

Материалы и методы. В работе использовали клетки MDCK, вирус гриппа A/chicken/Kurgan/05/2005 (A/H5N1), наночастицы АПС, нативные и модифицированные молекулы siRNA.

Результаты и обсуждение. Созданы уникальные нанокомплексы Si~NH_2/siRNA, состоящие из наночастиц АПС и иммобилизованных на них молекул siRNA, обеспечивающих соответственно проникновение в клетки и селективное взаимодействие с нуклеиновыми кислотами-мишенями. Противовирусную активность предложенных нанокомплексов исследовали на клетках MDCK, заражённых вирусом гриппа A/H5N1. Показано, что двухцепочечные молекулы siRNA в составе нанокомплексов, действующие по механизму РНК-интерференции, более эффективно подавляют репликацию вируса гриппа по сравнению с соответствующими одноцепочечными фрагментами РНК. Наиболее эффективный нанокомплекс, содержащий siRNA, нацеленную на выбранный участок 5-го сегмента мРНК вирусного генома, снижал репликацию вируса гриппа А в культуре клеток в 630 раз. Показано, что неагломерированные, растворимые в водных растворах наночастицы АПС являются малотоксичными, способными доставлять siRNA в клетки и защищать siRNA в составе нанокомплексов Si~NH_2/siRNA от гидролиза клеточными нуклеазами.

Заключение. Продемонстрирована высокая биологическая активность созданных нанокомплексов на примере селективного и высокоэффективного подавления репликации вируса гриппа A/chicken/Kurgan/05/2005 в клеточной системе.

Ключевые слова: *наночастицы аминопропилсиланола, нанокомплексы, siRNA, противовирусная активность, вирус гриппа A/H5N1*

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Introduction

Nucleic acid (NA) therapy offers unique opportunities to influence the genetic material of the cell. However, its effectiveness is limited by the instability of NAs in relation to cellular nucleases and their low ability to penetrate the cytoplasmic membrane, which makes it necessary to use different delivery systems [1].

Small interfering RNA (siRNA) is a promising type of RNA-based therapeutic agents because their mechanism of action is catalytic and each siRNA molecule can inactivate several target RNA molecules. siRNA molecules are intensively investigated as antiviral agents. L. Singh et al. presented a wide range of applications of nanoscale materials for the treatment of common viral infections [2]. For the clinical success of the proposed methods for the delivery of NA fragments into cells, safety and efficiency remain vital requirements. Various approaches have been proposed to address the problem of siRNA delivery, e.g., using viruses, cationic lipids, polymers and transport peptides. The successful use of bioconjugates of siRNA and N-acetylgalactosamines is also worth mentioning [3]. However, all methods have limitations for therapeutic use. A huge number of potential siRNA-based drugs have not undergone clinical trials because many factors (low efficiency of siRNA delivery to target cells, toxicity, degradation of siRNA by nucleases, filtration by kidneys, uptake by immune cells, off-target effects, low efficiency of penetration through hydrophobic cell membrane and release of siRNA from endosomes, etc.) limit the use of siRNA in biomedicine.

One of the most promising approaches to solving the problem of siRNA delivery into cells is the use of non-viral vectors based on nanoparticles (NPs) [2, 4, 5]. Different types of NPs have been used for delivery of siRNA. NPs consisting of cationic polymers (poly-L-lysine, polyamidoamine, polyethylenimine, and chitosan) or lipids are the most studied delivery methods [6, 7]. Given the wide variety of available materials, each with many potential modifications, the composition of the NPs can be optimized to deliver a specific type of RNA [8-10]. Delivery systems should fulfill a number of important requirements: they should increase the ability of RNA penetration into cells, provide effective protection of RNA from degradation by cellular nucleases, and have low toxicity.

Despite certain successes in the development of methods for the delivery of RNA fragments into cells, the problem of their delivery method still remains unsolved. Therefore, it is advisable to search for other methods to deliver siRNA into cells.

We have previously developed delivery systems for oligodeoxyribonucleotides and deoxyribozymes based on the use of titanium dioxide and aminopropylsilanol (APS) NPs for their effect on NA targets. It has been shown that DNA fragments within the created nanocomposites are site-specific and effectively affect target genes *in vitro* and *in vivo* [11–14].

The aim of this study is to determine the possibility of using non-agglomerated, water-soluble APS NPs for siRNA delivery into cells in the form of the Si~NH_2/siRNA nanocomplexes to effectively suppress replication of the influenza A/H5N1 virus.

Materials and methods

Reagents used in this study were from commercial suppliers: (3-aminopropyl)triethoxysilane, trypsin, penicillin, streptomycin (Sigma-Aldrich); DMEM medium (Dulbecco's modified Eagle's medium; Biolot); fetal bovine serum (Gibco), MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), NeoFroxx), and dimethyl sulfoxide (Component-Reactiv). Trypsin was used at a concentration of 2 μg/mL, penicillin and streptomycin at a concentration of 100 U/mL. Chicken erythrocytes, MDCK cells, and the influenza virus A/chicken/Kurgan/05/2005 (H5N1) strain were obtained from the collections of Vector.

Oligoribonucleotides and their derivatives were synthesized by the solid-phase method on an automated ASM-800 DNA/RNA synthesizer (Bioset) using an optimized protocol for a synthesis scale of 0.4 mmol. 2'-deoxy-, 2'-O-TBDMS-, and 2'-O-methyl-phosphoramidite (Glen Research) were used as monomers. Sulfurizing reagent II (Glen Research) was used to introduce the thiophosphate group. The concentration of oligonucleotides was determined spectrophotometrically by measuring their optical density in solution using a Shimadzu U-1800 spectrophotometer (Shimadzu).

Preparation of Si~NH_2 aminopropylsilanol nanoparticles and Si~NH_2/siRNA nanocomplexes

APS nanoparticles (Si~NH_2) were synthesized by hydrolysis of (3-aminopropyl)triethoxysilane, which was added dropwise to hot water, and the mixture was stirred at this temperature for 15 h followed by cooling to room temperature [15]. The pH value of the resulting solution (10.6) was adjusted to 7.5 using 1 M HCl. The concentration of the final Si~NH_2 solution (0.26 M) was evaluated by titration of amino groups using 1 M HCl. The reaction yield was 95–97%. Si~NH_2 NPs were studied by physicochemical methods, i.e., dynamic light scattering, ultraviolet, infrared spectroscopy, transmission and atomic force microscopy [15].

RNA molecules were immobilized on APS NPs [16] due to the electrostatic interaction between negatively charged internucleotide phosphate groups in oligoribonucleotides (p) and positively charged amino groups (NH_2) in NPs. The nanocomplexes with single- and double-stranded RNA (Si~NH_2/RNA and Si~NH_2/siRNA, respectively) were prepared by mixing RNA or siRNA with 0.26 M Si~NH_2 in water, provided that the NH_2/p ratio was 50 (we considered the number of

phosphate groups in only one strand). The size and zeta potential of the obtained APS nanoparticles and nanocomplexes with RNA molecules were measured by dynamic light scattering on a Zetasizer Nano ZS Plus device (Malvern).

Toxicity analysis of nanoparticles and nanocomplexes in MDCK cell culture

Samples in DMEM medium (Biolot; 0.1 mL at concentrations of 5–50 μM for siRNA or 5–50 mM for Si~NH_2) were added to wells of 96-well plates with MDCK cells. Cells in 0.1 mL of DMEM maintenance medium were used as a control. After incubation of cells for 2 days at 37ºC and 5% CO_2, the culture medium was removed and MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in PBS-D buffer (Dulbecco's phosphate-buffered saline; 0.075 mL, 1 mg/mL) was added to each well. Cells were incubated for 90 min at 37ºC, after which the dye solution was removed and dimethyl sulfoxide (0.1 mL) was added. After incubation for 10 min, the optical density in each well was measured on an Emax spectrophotometer (Molecular Devices) at a wavelength of 540 nm, which is an indicator of the number of viable cells in the monolayer.

The dependence of optical density on the concentration of the tested sample was presented in semi-logarithmic coordinates, and the 50% cytotoxic concentration (CC_{50}) of each sample was calculated using the SoftMaxPro-4.0 computer program.

Antiviral activity of nanocomplexes

A/chicken/Kurgan/05/2005 (H5N1) virus was grown in the allantois cavity of 10-day-old chicken embryos at 37ºC. Allantois fluid was collected 48 h after virus inoculation and stored at –80ºC. MDCK cells were seeded at a rate of 10^5 cells/mL in DMEM nutrient medium containing 10% fetal bovine serum (Gibco) into 96-well plates (100 μL/well) and incubated at 37ºC, 5% CO_2, and 100% humidity. After reaching ~80% monolayer, the medium was removed and samples of Si~NH_2, Si~NH_2/RNA, and Si~NH_2/siRNA were added to the wells at a concentration of 0.5 mM (for Si~NH_2, corresponding to 0.5 μM for RNA or siRNA) in 100 μL of DMEM medium. The control sample was the same medium without the nanocomplexes. Oseltamivir at a concentration of 10 μg/mL was used as a reference drug.

The concentration of the Si~NH_2/siRNA nanocomplexes was varied in the range of 0.01–1.00 μM (for siRNA) in experiments that involved the investigation of their dose-dependent antiviral activity.

Cells were incubated in the presence of the samples at 37ºC, 5% CO_2, and 100% humidity for 4 h followed by washing the cells with the same medium. Cells were then infected with the A/H5N1 virus in trypsin-containing (2 μg/mL) DMEM medium (100 μL in each well) at an infection multiplicity of 0.01 50% tissue culture infectious dose ($TCID_{50}$) per 1 mL. After virus adsorption for 1 h at room temperature, the medium containing virus was removed, cells were washed with trypsin-free DMEM medium, and the same medium containing trypsin was added to each well (100 μL). After incubation for 48 h, serial 10-fold dilutions (10^{-1} to 10^{-8}) of the culture fluid containing virus from each well were applied to MDCK cells with repeated incubation for 48 h for further evaluation of the virus titer. The presence of cytopathic action was recorded under the microscope and in hemagglutination reaction with 1% suspension of chicken erythrocytes. The virus titer was expressed in terms of lg $TCID_{50}$/mL. To evaluate the dependence of virus inhibition on the concentration of nanocomplexes, the percentage of inhibition of virus production was calculated using the formula: $(A – B)/A$, where A is the virus titer in control (without sample) in $TCID_{50}$/mL; B is the virus titer in experiment (with sample) in $TCID_{50}$/mL.

Statistical analysis

Statistical analysis was performed using the Statistica v. 12 program (StatSoft Inc.). Virus titer in control and experiment (without or with experimental samples, respectively) was calculated using the Spearman–Kerber method and expressed as lg $TCID_{50}$/mL. Differences between the results with experimental and control samples were considered significant at $p \leq 0.05$.

Results and discussion

Silica NPs are considered as promising carriers for delivery of NAs into cells [17]. Most often, amine-modified Si-NPs are used for immobilization of NAs and their fragments. We synthesized non-agglomerated APS NPs (hydrodynamic diameter — ~1 nm, zeta potential — ~10 mV) [15].

The small size of Si~NH_2 particles provide water-soluble preparations. Characterization of APS NPs using physicochemical methods is described in our previous work [15]. It is shown that the obtained NPs are not prone to agglomeration and can be stored for several months.

Si~NH_2/RNA and Si~NH_2/siRNA nanocomplexes were obtained by electrostatic interaction between negatively charged inter-nucleotide phosphate groups in RNA and siRNA and positively charged protonated amino groups in Si~NH_2 NPs. The addition of negatively charged siRNA molecules to the NPs leads to a change in zeta potential from ~(+10 mV) to ~(–30 mV) and particle size from ~1 nm to ~200 nm, thus indicating the formation of nanocomplexes.

As a target for RNA and siRNA, we chose segment 5 of the influenza A virus encoding a nucleoprotein that plays a key role in the incorporation of the viral genome into the cell nucleus of an infected organism, thus facilitating further replication and assembly of vi-

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ral particles [18]. A region near the 3' end of this segment starting at nucleotide 1496, which is conserved vulnerable to siRNA action [19–21], was selected for siRNA exposure.

We synthesized two native (RNA_1 and RNA_2) and eight modified (RNA_3–RNA_{10}) oligoribonucleotides containing TT at the 3' end (**Table 1**). RNA_5 and RNA_6 contain, in addition to TT, 2'-OMe groups at the U*A site in the center of the strands. The presence of the 2'-O-Me group in this site increases resistance to serum nucleases, thereby maintaining the interference ability of siRNA [22] and providing long-term suppression of target gene expression [23]. The RNA_7 strand contains 2'-O-Me groups at all positions and 2 internucleotide thiophosphate groups at both ends. The RNA_8 strand differs from RNA_7 by the presence of three consecutive 2'-F groups at positions 9–11 from the 5' end. Oligoribonucleotides RNA_2, RNA_4, RNA_6, and RNA_8 are sense strands, and RNA_1, RNA_3, RNA_5, and RNA_7 are antisense strands directed to (–)RNA and (+)RNA of the viral genome, respectively.

The synthesized RNAs were used to obtain the Si~NH_2/RNA nanocomplexes based on non-agglomerated APS NPs. Their biological activity was investigated on the example of suppression of the A/H5N1 virus replication in infected MDCK cells at a multiplicity of infection of 0.01 $TCID_{50}$/cell. The results show that all single-stranded oligoribonucleotides (both sense and antisense strands) in the Si~NH_2/RNA nanocomplexes suppressed replication of the influenza A/H5N1 virus by 0.7–1.3 orders of magnitude (5–20 times) (**Fig. 1**, columns 1–8).

From the four studied sense strands, we chose RNA_4 containing deoxydinucleotide TT at the 3' end for protection from exonucleases, as the most active one and formed four siRNA molecules with all possible antisense strands ($RNA_{1/4}$, $RNA_{3/4}$, $RNA_{5/4}$, and $RNA_{7/4}$). All siRNAs are duplexes with the same nucleotide sequence and different modification of nucleoside units. The efficiency of the investigated siRNAs was significantly higher compared with single-stranded oligoribonucleotides (Fig. 1). The Si~NH_2/siRNA nanocomplexes suppressed the A/H5N1 virus replication by 2.3–2.8 orders of magnitude (200–630 times).

The main reason for the difference in the effectiveness of siRNA and oligoribonucleotides is the different mechanism of their action. It is known that oligodeoxynucleotides and siRNAs suppress the functions of target RNAs through complementary interactions with the target RNA with its subsequent degradation by cellular RNases (RNase-H1 and AGO2, respectively) [24–26], which eventually leads to the loss of target RNA functions. Single-stranded RNA fragments can form complementary complexes with the target RNA but do not cause degradation of the target RNA. The siRNA mo-

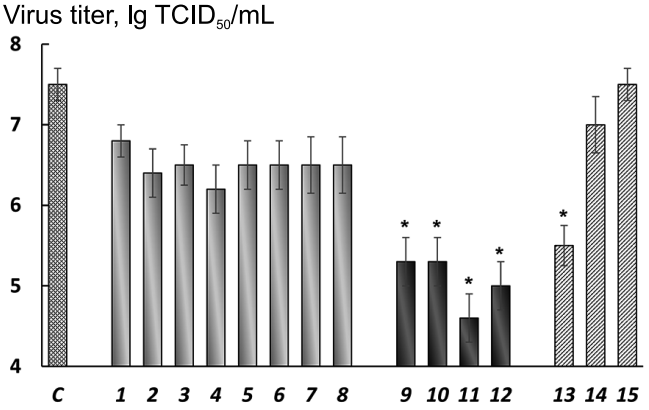

Fig. 1. Titers of A/chicken/Kurgan/05/2005 (H5N1) virus in MDCK cells after their incubation of with Si~NH_2/RNA and Si~NH_2/siRNA nanocomplexes.

C, virus control without samples. Si~NH_2/RNA nanocomplexes containing RNA_1–RNA_8 (*1–8*), concentration of RNA in nanocomplexes is 0.5 μM; Si~NH_2/siRNA nanocomplexes containing $siRNA_{1/4}$, $siRNA_{3/4}$, $siRNA_{5/4}$ and $siRNA_{7/4}$ (*9–12*), concentration of siRNA in nanocomplexes is 0.5 μM per one strand; ozeltamivir (*13*), 10 μg/mL or 32 μM; Si~NH_2 (*14*), 0.5 mM; Si~NH_2/$siRNA_{9/10}$, nanocomplex containing unspecific $siRNA_{9/10}$ *(15)*. MOI, 0.01 $TCID_{50}$/cell. The presented average values, standard deviations and differences in the titer values of the virus are calculated using the Spearman–Kerber method. Asterisks designate the difference between the control and the titer values of the virus obtained under the action of the studied series of nanocomplexes, at $p < 0.05$.

Table 1. Oligoribonucleotides used in this study

RNA chain		Nucleotide sequence, 5'-3'
RNA_1	Antisense	CUCCGAAGAAAUAAGAUCC
RNA_2	Sense	GGAUCUUAUUUCUUCGGAG
RNA_3	Antisense	CUCCGAAGAAAUAAGAUCCTT
RNA_4	Sense	GGAUCUUAUUUCUUCGGAGTT
RNA_5	Antisense	CUCCGAAGAAAU*AAGAUCCTT
RNA_6	Sense	GGAUCUU*AUUUCUUCGGAGTT
RNA_7	Antisense	$C^*_{PS}U^*_{PS}C^*C^*G^*A^*A^*G^*A^*A^*A^*U^*A^*A^*G^*A^*U^*C^*C^*_{PS}T_{PS}T$
RNA_8	Sense	$G^*_{PS}G^*_{PS}A^*U^*U^*U^{\#}A^*U^{\#}U^{\#}C^*U^*U^*C^*G^*G^*A^*G^*_{PS}T_{PS}T$

Note. *2'-O-methyl group; #2'-fluoro group; PS — internucleotide thiophosphate group; symbol d for deoxyribotimidine has been omitted.

Table 2. Dependence of antiviral activity of Si~NH_2/$siRNA_{5/4}$ nanocomplex on siRNA concentration with MOI 0.01 $TCID_{50}$/cell

Concentration of $siRNA_{5/4}$ in nanocomplex, μM	Infection titer of influenza A virus $lgTCID_{50}$/mL	Inhibition of influenza A virus replication, %
1,00	4,50	99,99
0,50	5,75	99,82
0,10	7,00	97
0,05	7,50	90
0,01	7,50	90
Virus control	8,50	–

lecule first binds to the RISC complex, then the sense (passenger) strand is removed. AGO2 nuclease and the remaining antisense strand within the RISC complex find the target RNA, and AGO2 cleaves it. AGO2 retains the antisense strand for some time as part of the RISC complex for further reactions [27–29].

It is known that siRNAs are rapidly hydrolyzed by cellular nucleases, and various modifications are used to protect against them. It should be noted that even minimally modified $siRNA_{1/4}$ and $siRNA_{3/4}$ delivered into the cells as part of nanocomplexes with NPs were very effective in suppressing virus replication (by 2.3 orders of magnitude, ~200 times). This implies that APS NPs protect siRNA from cellular nucleases. The most active Si~NH_2/$siRNA_{5/4}$ nanocomplex suppressed virus replication by ~3 orders of magnitude.

The antiviral activity of the studied Si~NH_2/siRNA nanocomplexes was comparable to that of oseltamivir (the most commonly used comparison drug in studies of effects on the influenza A virus) but at a much lower concentration of the active component (0.5 μM for siRNA and 32 μM for oseltamivir). The Si~NH_2 NPs did not inhibit virus replication, as would be expected.

The most active Si~NH_2/$siRNA_{5/4}$ nanocomplex is characterized in more detail. We evaluated the effect of siRNA, Si~NH_2 NPs in the free state and as part of the Si~NH_2/$siRNA_{5/4}$ nanocomplex on the survival of uninfected MDCK cells (**Fig. 2**). Unbound $siRNA_{5/4}$, as expected, was nontoxic in the concentration range investigated. The cytotoxicity of the Si~NH_2/$siRNA_{5/4}$ nanocomplex coincides with the toxicity of Si~NH_2 NPs. Consequently, it can be concluded that the cytotoxicity of nanocomplexes is determined by the toxicity of their constituent NPs. The cytotoxic concentration of the drug, at which 50% of cells in the uninfected monolayer are destroyed, determined from the data in Fig. 2, was 38 mM per Si~NH_2 and 38 μM per siRNA.

Table 2 summarizes the results of suppression of the influenza virus production depending on the concentration of the Si~NH_2/$siRNA_{5/4}$ nanocomplex in the cell culture.

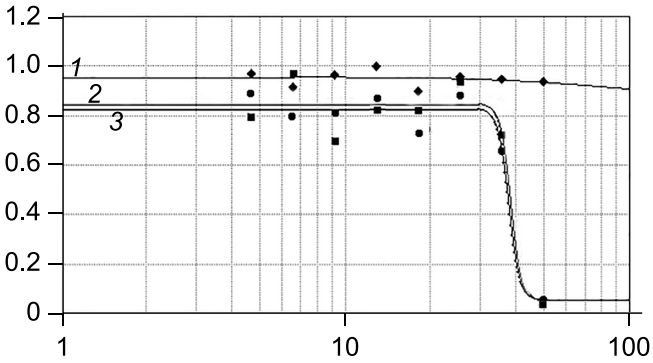

Fig. 2. Viability of MDCK cells when treated with siRNA and Si~NH_2 samples in the free state and as part of the Si~NH_2/siRNA nanocomplex.

1, $siRNA_{5/4}$; *2,* Si~NH_2; *3,* Si~NH_2/$siRNA_{5/4}$. The x-axis shows the concentration of siRNA in the free state or as part of a nanocomplex (μM) and Si~NH_2 nanoparticles in the free state or as part of a nanocomplex (mM). The y-axis shows optical density of the MTT solution.

It was shown that in the concentration range of $siRNA_{5/4}$ in the Si~NH_2/$siRNA_{5/4}$ nanocomplex from 0.01 to 1.00 μM, the suppression of the influenza virus production was 90.00–99.99%.

Conclusion

The results indicate that APS NPs can be used to deliver siRNA into cells as part of the Si~NH_2/siRNA nanocomplexes. The cytotoxicity of the Si~NH_2/$siRNA_{5/4}$ nanocomplex is determined by the toxicity of Si~NH_2 NPs. The cytotoxic concentration of the drug, at which 50% of cells in the uninfected monolayer are destroyed, was 38 mM per Si~NH_2 and 38 μM per siRNA. The proposed siRNA-containing nanocomplexes targeting a selected region of the 5th segment of the viral genome mRNA were successfully used to suppress the A/H5N1 virus production in a cellular system. The most effective Si~NH_2/$siRNA_{5/4}$ nanocomplex reduced the replication of the influenza A virus in cell culture by ~3 orders of magnitude.

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Original Study Article
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Time series analysis of dengue incidence in Bandung City, Indonesia using a ARIMA model

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Abstract

Background. Dengue is a public health problem that leads to death. This disease is necessary to monitor to reduce its impact on the community.

Purpose. This study aims to forecast the incidence of dengue in Bandung City using historical data from 2014 to 2023.

Method. This retrospective observational study examined dengue incidence in Bandung City from 2014 to 2023, secondary data were processed and analysed using Autoregressive Integrated Moving Average (ARIMA) model to forecast dengue incidence.

Results. The best model generated is ARIMA (3,0,3), Mean Absolute Percentage Error (MAPE = 33,3437) and Akaike Information Criterion (AIC = 0,1489). Based on the model, the peak of dengue cases is estimated to occur in September 2024 (320 cases).

Conclusion. The peak incidence of dengue in Bandung City will occur in September 2024. Hence the need for vector control efforts in several sub-districts and increasing efforts to prevent and control dengue.

Keywords: *Dengue incidence, Dengue forecast, ARIMA model, outbreak prediction, Indonesia*

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Оригинальное исследование
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Анализ временны́х рядов заболеваемости денге в городе Бандунг, Индонезия, с использованием модели ARIMA

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Аннотация

Введение. Вирус денге является проблемой общественного здравоохранения, которая приводит к смерти. Это заболевание необходимо контролировать, чтобы уменьшить его воздействие на общество.

Цель исследования — спрогнозировать заболеваемость денге в городе Бандунг, используя данные с 2014 по 2023 г.

Метод. В данном исследовании изучалась заболеваемость денге в городе Бандунг с 2014 по 2023 г., вторичные данные обрабатывали и анализировали с помощью модели авторегрессии скользящего среднего (ARIMA) для прогнозирования заболеваемости денге.

Результаты. Лучшей моделью является ARIMA (3,0,3), средняя абсолютная ошибка в процентах (MAPE = 33,3437) и информационный критерий Акаике (AIC = 0,1489). Исходя из модели, пик заболеваемости денге приходится на сентябрь 2024 г. (320 случаев).

Выводы. Пик заболеваемости денге в городе Бандунг придётся на сентябрь 2024 г. Отсюда следует, что необходимо проводить мероприятия по борьбе с переносчиками инфекции в нескольких подрайонах и наращивать усилия по профилактике и борьбе с денге.

Ключевые слова: *заболеваемость денге, прогноз заболеваемости денге, модель ARIMA, прогноз вспышек, Индонезия*

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

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Introduction

Dengue is a disease transmitted by the Aedes aegypti and Aedes albopictus mosquitoes, caused by the dengue virus. It is more common in tropical and subtropical climates[1]. In 2013, approximately 3.6 billion people and 40% of the global population resided in areas at risk of dengue or endemic to the disease. It is estimated that 400 million individuals contracted the dengue virus annually, resulting in 100 million dengue cases and approximately 21,000 deaths[2]. A total of more than 100 countries worldwide have been identified as being endemic to dengue. The Americas, Southeast Asia and the Western Pacific are the most severely affected regions by this disease. Seventy percent of the disease burden is concentrated in Asia. South and Southeast Asia are the regions with the highest number of cases, with an estimated 1.3 billion people residing in dengue-endemic areas across 10 countries in Southeast Asia. Among the 30 countries with the highest endemicity rate in the world, India, Indonesia, Myanmar, Sri Lanka and Thailand are included[3]. Indonesia is one of the countries contributing the most cases of dengue in the world, with the number of cases continuing to increase. In 2021, there were 73,518 cases, which increased to 143,266 in 2022. In 2023, there were 114,720 cases, resulting in 894 deaths[4]. Bandung City is one of the regions in Indonesia that contributes the highest incidence of dengue. In 2021, the incidence of dengue in Bandung City was recorded at 3,743 cases, increasing to 5,205 cases in 2022 and resulting in 10 deaths[5].

The early identification of dengue outbreaks has the potential to enhance vector control efforts and permit public health authorities to implement proactive measures to prevent the spread of the disease [1]. A multitude of mathematical models have been employed to predict dengue incidence. Time series models are the most prevalent, as evidenced by studies conducted in Thailand [2], Singapore [3], Brazil [4] and China [5]. This model employs a combined approach between environmental and biological factors in order to forecast the risk of transmission and the magnitude of potential outbreaks. Furthermore, this model can integrate the complex interactions between environmental and biological factors that affect dengue transmission [6]. The models presented in some of these studies indicate that time series analysis is an invaluable tool

[1] World Health Organization. Dengue and severe dengue 2022. URL: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

[2] Centers for Disease Control and Prevention. Why is Dengue a Global Issue. URL: https://www.cdc.gov/dengue/training/cme/ccm/page51440.html

[3] World Health Organization. Dengue in the South-East Asia. URL: https://www.who.int/southeastasia/health-topics/dengue-and-severe-dengue

[4] Ministry of Health of the Republic of Indonesia. Latest information on dengue up to week 8 2024. URL: https://p2pm.kemkes.go.id/publikasi/infografis/informasi-terkini-dbd-hingga-minggu-ke-8-2024

[5] Health Office of Bandung City. Health Profile of Bandung City in 2022. URL: https://dinkes.bandung.go.id/download/profil-kesehatan-2022

for enhancing our understanding of dengue transmission dynamics and for the design of effective preventive measures to control the disease.

Time series analysis is a valuable tool in public health and infectious disease surveillance [7]. Autoregressive Integrated Moving Average (ARIMA) model is a time series modelling technique that has been widely used to predict and forecast data with seasonal patterns. The model has proven effective in modelling and forecasting dengue incidence, revealing temporal patterns, and predicting future trends. The ARIMA model is versatile and has many practical applications in dengue prevention and control. The model is capable of identifying temporal patterns, thereby guiding targeted vector control measures during high-risk periods. Furthermore, the ARIMA model assists in developing an early warning system for dengue outbreaks, thereby improving the efficiency and effectiveness of vector control campaigns. These analyses provide insights into dengue transmission patterns and the impact of environmental and social factors on disease incidence [6]. The application of ARIMA modelling has facilitated a deeper understanding of temporal patterns, thereby enabling more effective prevention and control of outbreaks. This approach has been successfully employed in Brazil, Mexico, Singapore, Sri Lanka and Thailand [8].

The incidence of dengue cases in Indonesia is rising year on year, yet the efforts made to combat the disease are not yet optimal. Furthermore, the current treatment approach has not been proven effective in overcoming dengue, with the focus being on mitigating complications and reducing the severity of symptoms instead [9]. Consequently, there is a pressing necessity to implement effective prevention strategies. The ARIMA model is frequently employed in case forecasting research and provides insight into dengue epidemiology. However, in this study, the author sought to offer novel insights specific to the distinctive dynamics of dengue transmission in Bandung City. These insights can assist policymakers in developing early vigilance efforts and implementing dengue epidemiological surveillance, particularly with regard to place and time. The objective of this study is to forecast the incidence of dengue in order to facilitate more efficient dengue prevention and control efforts.

Materials and methods

Study design

This retrospective observational study was conducted using historical data of dengue cases in Bandung City, Indonesia from 2014 to 2023.

Study area

The study area of this research is Bandung City which is the capital of West Java Province, Indonesia, and is the third largest city in Indonesia. located at 107º36‘East, 6º55’ LS, and covers an area of 167.3/km². In 2023 the population of Bandung City is 2,569,107 people with a population density of approximately 15,000 people per square kilometre. Bandung City was chosen as the research location because it is a dengue endemic area and recorded a significantly higher amount of dengue cases from 2014 to 2023 compared to other regions.

Data collection

This study used secondary data from the Bandung City Health Office. The Disease Prevention and Control Unit of Bandung City Health Office provided monthly data on dengue incidence in Bandung City from January 2014 to December 2023. The monthly dataset contains a total of 120 monthly observations. The author used dengue cases covering 2014–2023 due to the availability of the dataset recorded from Bandung City Health Office. Dengue incidence data is something that must be reported in Bandung City routinely. The data available at the Bandung City Health Office are transferred data from community health center, hospitals and clinics, so this data is sufficient to describe the actual incidence.

Data management and statistical analysis

The forecasting data analysis method used R Studio software. The temporal pattern of dengue incidence in Bandung City was analysed using Autoregressive Integrated Moving Average (ARIMA), a statistical modelling approach that has been widely used for time series analysis. This approach was popularised by Box and Jenkins. During the study period, monthly dengue incidence cases were used to create an ARIMA time series analysis. In general, when dealing with data that does not show seasonal patterns, the model used is ARIMA (p, d, q). The parameters of the ARIMA model are as follows: p is the autoregressive (AR) number, which determines how many time periods are used to predict the current period. This parameter is determined from the partial autocorrelation function (PACF) diagram, d: the number of differentials taken for the static average and the parameter q represents the moving average (MA) number, which takes into account the deviation of the data series from the average of the series over a number of time periods to predict the current time period. These parameters are determined from the autocorrelation function (ACF) diagram. The evaluation of time series models includes the use of Akaike Information Criteria (AIC). Lower normalised AIC values are considered to be more favourable. Once the optimal model had been identified, the author proceeded to perform forecasting for 2024.

Ethical clearance

Ethical clearance and permission for this study was obtained from the Health Research Ethics Committee of Bhakti Kencana University with No. 079/09. KEPK/UBK/VII/2023.

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Results

Figure 1 shows the map of Bandung City Area along with dengue incidence maps from 2014 to 2023. These maps highlight the dengue incidence based on 30 sub-districts. The highest cases occurred in 2022 at 5,205 cases. The highest number of cases is in Bojongloa Kidul Sub-district (299 cases), and the lowest number of cases is in Bandung Wetan Sub-district (65 cases). Meanwhile, the lowest number of cases occurred in 2017 at 1,786 cases. The highest number of cases was in Buah batu sub-district (134 cases) and the lowest number was in Cicadap sub-district (12 cases).

This study uses a dataset consisting of monthly dengue events in Bandung City from January 2014 to December 2023, which shows a random or non-seasonal pattern. The observed monthly dengue case series showed a stationary pattern as shown in **Figure 2**, *a*. To assess the stationarity of the data, the Dickey-Fuller test was performed and showed the data was stationary ($p = 0.01$). In the ACF plot, the value drops exponentially as it approaches 0 in Fig. 2, *b*. While the PACF that exceeds the maximum limit is at Lag 0, 1 and 3, then the appropriate model is AR = 3 seen in Fig. 2, *c*.

After identifying the model, we estimated the ARIMA model to determine the significant model. **Table 1** shows that the ARIMA models with significant coefficient estimates are ARIMA (3,0,1), ARIMA (3,0,2) and ARIMA (3,0,3).

The ARIMA models with significant coefficient estimates are ARIMA (3,0,1), ARIMA (3,0,2), and ARIMA (3,0,3). Diagnostic checking is done to ensure the best ARIMA model. The ACF plot of residuals does not exceed the boundary line for lag > 0, and the Ljung-box test shows that all values are above the boundary line, so the ARIMA (3,0,1) model is appropriate, otherwise there is no autocorrelation (**Fig. 3**, *a*). ACF plot of residuals exceeds the boundary line for lags > 0. The Ljung-box test shows that all values are above the boundary line, so the ARIMA (3,0,2) model is appropriate, otherwise there is no autocorrelation (Fig. 3, *b*). ACF plot of residuals exceeding the boundary line for lags > 0. The Ljung-box test shows that all values are above the boundary line, so the ARIMA (3,0,3) model is appropriate, otherwisethere is no autocorrelation (Fig. 3, *c*).

The ARIMA (3,0,1), ARIMA (3,0,2) and ARIMA (3,0,3) models are all good, so to determine the best ARIMA model, the Mean Absolute Percentage Error (MAPE), and Akaike information criterion (AIC) values are used. **Table 2** shows the values of Mean Absolute Error (MAE), Root Mean Squared Error (RMSE), and Mean Absolute Percentage Error (MAPE), and Akaike information criterion (AIC). The most suitable model forecast result is the ARIMA (3,0,3) model, which can be seen from the MAPE value of 33.3437 and the AIC value of 0.1489.

Table 1. Estimation of ARIMA Model

Model	AR *p*-value	MA *p*-value
ARIMA (0,0,1)	–	Significant
ARIMA (0,0,2)	–	Significant
ARIMA (0,0,3)	–	Significant
ARIMA (1,0,0)	Significant	–
ARIMA (1,0,1)	Significant	Not significant
ARIMA (1,0,2)	Significant	Not significant
ARIMA (1,0,3)	Significant	Not significant
ARIMA (2,0,0)	Significant	–
ARIMA (2,0,1)	Significant	Not significant
ARIMA (2,0,2)	Not significant	Not significant
ARIMA (3,0,0)	Significant	Not significant
ARIMA (3,0,1)	Significant	Significant
ARIMA (3,0,2)	Significant	Significant
ARIMA (3,0,3)	Significant	Significant

ARIMA model (3,0,3) to forecast dengue incidence from January 2024 to December 2024, with a MAPE value of 33.3437 or 3.33%. The observed and forecasted values of dengue incidence for 2024 are presented in **Fig. 4**. The trend of dengue cases in Bandung City from January to December 2024 tends to increase.

The results of the forecast of dengue incidence in Bandung City in 2024 (**Table 3**), we found that the highest number of cases occurred in September 2024 which was 320 and the lowest in January 2024 which was 217 cases This finding is quite similar to the pattern of dengue incidence in observational data, so this model is sufficient to describe the forecast of dengue incidence in 2024.

Discussion

The incidence of dengue in Bandung City has consistently posed a significant public health concern in recent years. The city's dengue incidence rate is notably higher than that of other cities and districts, with a high mortality rate. Dengue incidence is typically highest during the rainy season [10], with population

Table 2. Mean Absolute Percentage Error (MAPE), and Akaike information criterion (AIC) on ARIMA Model

Model	RSME	MAE	MAPE	AIC
ARIMA (3,0,1)	114.0923	75.344	34.08223	0,1492
ARIMA (3,0,2)	111.3093	74.16202	33.87018	0,1490
ARIMA (3,0,3)	110.0493	73.42905	33.3437	0,1489

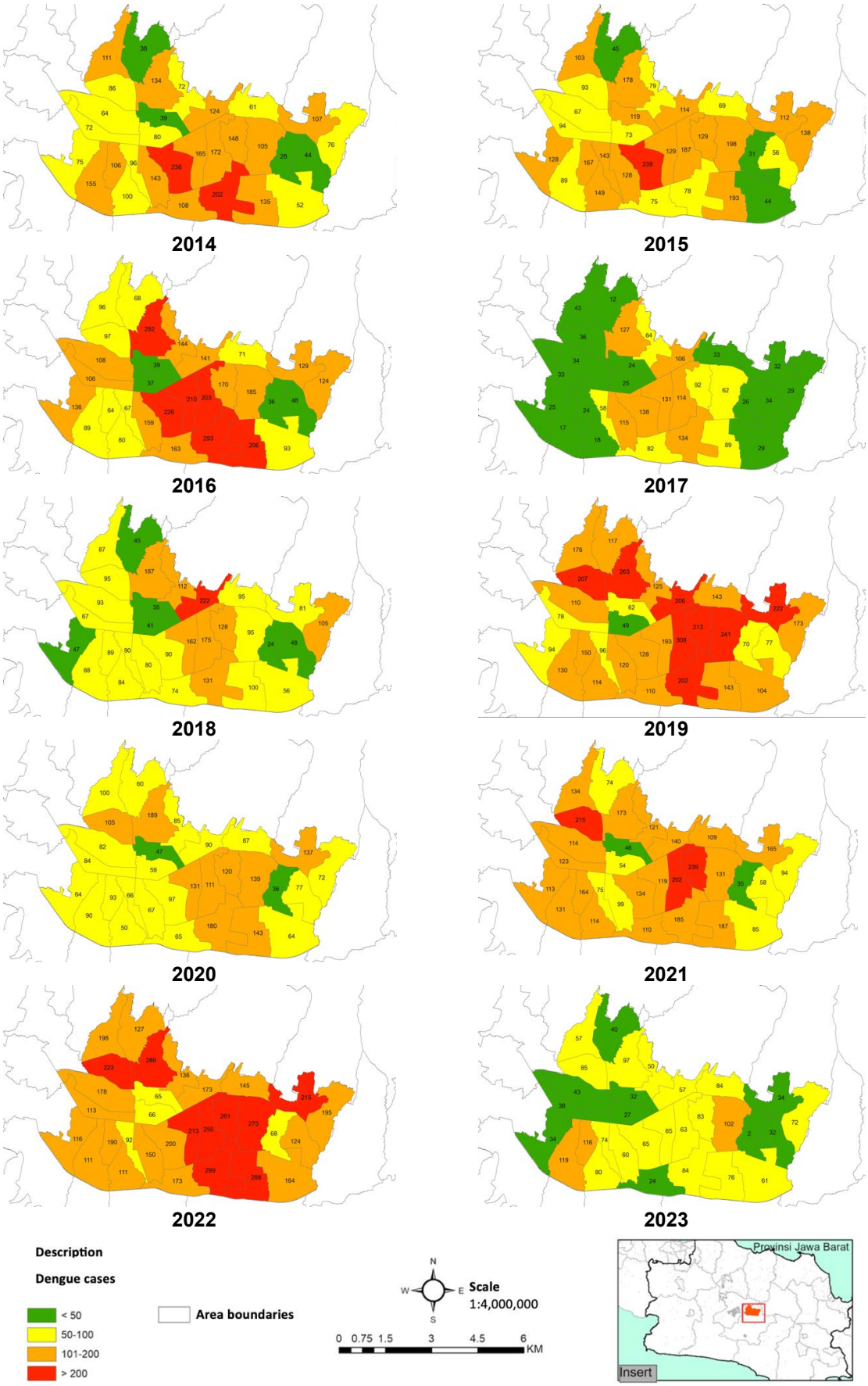

Fig. 1. Dengue incidence in Bandung City in 2014–2023.

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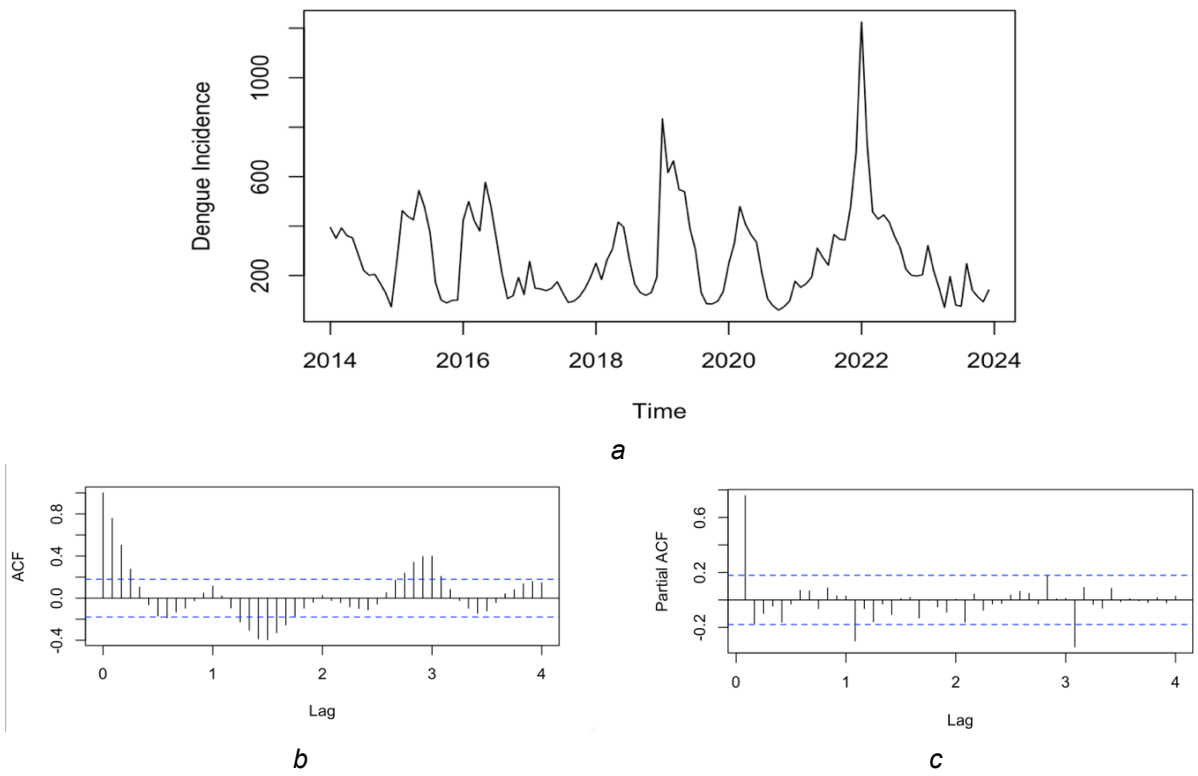

Fig. 2. Dengue incidence based on January 2014 to December 2023 in Bandung City (*a*), autocorrelation function (*b*), partial autocorrelation function (*c*).

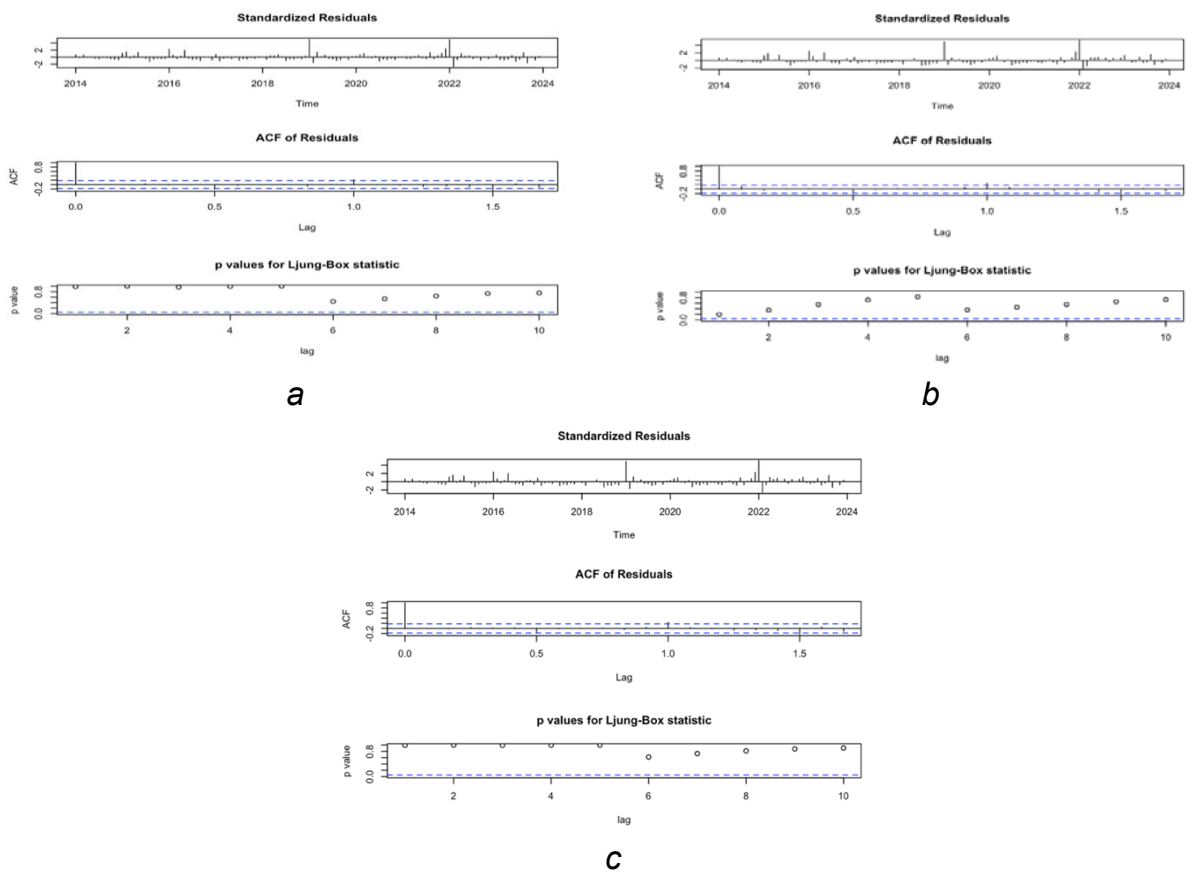

Fig. 3. Diagnostic checking of ARIMA Model (3,0,1) (*a*), ARIMA Model (3,0,2) (*b*), RIMA Model (3,0,3) (*c*).

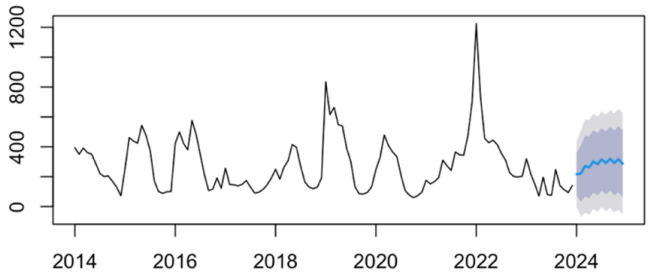

Fig. 4. Observed and predicted dengue incidence from ARIMA model (3,0,3) in 2024 in Bandung City.

Table 3. Prediction of dengue fever cases in Bandung City in 2024 obtained from the ARIMA model (3,0,3)

Month	Cases
Jan-24	217
Feb-24	221
Mar-24	272
Apr-24	263
May-24	303
Jun-24	285
Jul-24	316
Aug-24	293
Sep-24	320
Oct-24	293
Nov-24	318
Dec-24	289

density also playing a role [11]. The Indonesian government has implemented dengue prevention and control efforts, including the eradication of mosquito nests [12]. Case experience, such as the ARIMA technique, plays an important role in the conduct of dengue prevention and control planning. This study employed the ARIMA technique to forecast dengue incidence in Bandung City. The findings of this study can serve as a source of information for the government and researchers in preparing and responding to dengue outbreaks in Indonesia.

This study utilises monthly dengue incidence data from January 2014 to December 2023. The SARIMA model was employed to forecast the number of dengue events in 2024. The accuracy of the SARIMA model is contingent upon the quality and availability of data, as well as the selection of appropriate model parameters and assumptions. In this study, the observation data is stationary. This study demonstrates that large data sets facilitate the generation of more accurate models and forecasts. The best model for forecasting Bandung City cases is the ARIMA model (3,0,3), which provides the most accurate forecast of dengue incidence. ARIMA models have been widely used in Southeast Asia, especially in Malaysia, Vietnam, Myanmar and Thailand. Some of these countries have used the ARIMA model to forecast dengue incidence effectively [13–16]. Another study in Indonesia posited that the ARIMA model can assist in the prediction of dengue cases and can be utilized by the government to design effective public health measures to prevent and control dengue incidence, particularly at the outset of an outbreak [17]. A study conducted Indonesia found that the SARIMA model accurately predicts monthly dengue cases, thereby supporting the development of an early warning system for dengue outbreaks [18].

The findings in Sri Lanka indicate that the ARIMA model has demonstrated its capacity in effectively forecasting weekly dengue cases. This makes it a viable proposition for forecasting weekly dengue incidence in the short term. The model can be utilised to improve the Ministry of Health's preparedness and response strategies, ultimately contributing to the proactive management of dengue outbreaks [19]. In Bandung City and Indonesia, the dengue incidence forecasting model can help in improving the existing strategies in preventing and controlling dengue disease. Furthermore, the prediction of cases can facilitate the response to outbreaks that do occur, given that Bandung City is an endemic area and that frequent outbreaks are a regular occurrence. Therefore, forecasting models are required.

The findings of this study have significant implications for dengue prevention policies and practices in Bandung City. The use of the ARIMA (3,0,3) forecasting model can provide important information about the appropriate strategies in preventing and controlling dengue incidence, including mosquito nest eradication measures and vector control. It is predicted that dengue incidence will increase in September 2024, which coincides with the beginning of the rainy season. This finding is consistent with research in Nepal which states a seasonal pattern of dengue incidence, with the development of cases in September, reaching the highest point in September-October. Dengue cases peak in months with the highest temperature and rainfall [20]. The spatial map of dengue incidence in Bandung City from 2014 to 2023 is quite varied, with several sub-districts exhibiting a high number of cases each year. Sub-districts with a high number of cases are areas with a high population density. Findings in Sri Lanka and Brazil have indicated that the spread of dengue vectors is due to demographic factors such as population density [21, 22]. Other studies have identified higher dengue incidence rates in certain areas due to climatic variations, socioeconomic status, urbanisation, and vector control efforts [23, 24].

The predictive accuracy of this model can be enhanced by incorporating the potential impact of climatic variables, such as temperature, humidity, precipitation and wind speed, on dengue transmission [25, 26]. These climatic factors are known to play a significant role in dengue transmission [27], and therefore, it is essential to include them in future research to enhance the predictive capacity of the model and facilitate a deeper understanding of disease mechanisms and the develop-

ment of effective public health interventions. Bandung City is situated at an average altitude of 700 metres above sea level, which is conducive to the proliferation of Aedes aegypti mosquitoes. Research conducted in Colombia indicates that the Aedes aegypti mosquito is more prevalent at altitudes below 1,000 metres [28]. Furthermore, the seasonal increase in dengue incidence in Bandung City is attributed to the density of mosquito larvae. Previous findings have indicated that the entomological index, which is defined as the density of mosquito larvae, plays a significant role in the increase of dengue incidence in Bandung City [29].

Given the limitations of this study, including the potential influence of climatic factors, population density, altitude and high population mobility, it is crucial to develop a model to describe dengue case patterns in future studies. Overall, this study has significant implications for the public health of Bandung City and Indonesia. It is imperative to implement prevention efforts at the beginning of the peak cases to avoid the occurrence of dengue outbreaks.

Conclusion

This study presents a ARIMA model for forecasting dengue incidence in Bandung City, Indonesia. Monthly confirmed dengue events in Bandung City were obtained from 2014 to 2023 for this study, with the objective of forecasting dengue disease outbreaks in the early phase and enabling rapid response. The ARIMA model (3,0,3) proved to be the most accurate in forecasting future dengue incidence. The model predicts the peak of dengue cases in September 2024 with an estimated 320 cases. This model will be useful for dengue epidemiological surveillance and for policy makers in improving dengue prevention and control efforts. The spatial map shows that certain sub-districts had very high dengue incidence from 2014 to 2023, which emphasises the necessity for targeted intervention in high-risk areas for vector control. The incorporation of climatic variables, in conjunction with other factors such as population density, altitude and population mobility, is essential for the generation of more precise disease incidence forecasts. Consequently, these variables should be incorporated into the development of models designed to describe future dengue incidence patterns.

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ORIGINAL RESEARCHES

Original Study Article
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Molecular detection of *Streptococcus agalactiae* in pregnant women and percentage of vertical transmission to their neonates in Babylon province

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Abstract

Introduction. *Streptococcus agalactiae* are gram-positive, non-motile and encapsulated cocci. On blood agar, they produce an narrow zone of beta-haemolysis. This pathogen causes invasive bacterial diseases in newborns, including sepsis, meningitis, septicaemia, and pneumonia, when transmitted from infected mothers. Since *S. agalactiae* is a pathogen of primary concern for public health, this research has been conducted on it.

The **objective** of the study is the isolation and molecular detection of virulence gene of *S. agalactiae* group B (GBS), and evaluation of the percentage of mother-to-child transmission of the pathogen.

Materials and methods. A prospective cohort study was designed that included 300 pregnant women who were at more than 35 weeks of pregnancy. The gynaecologist collected 300 vaginal swabs from all participants in this study and followed up on all GBS-positive pregnant women after delivery to take swabs from their neonates. Traditional microbiological and molecular approaches were used to study isolated bacteria.

Result. Sixty (20%) of three hundred pregnant women and 16 (26.6%) of their newborns were enrolled in this study. GBS was detected via culture methods and was confirmed by PCR with primers employed for the detection of *atr* gene (housekeeping gene). Positive isolates were 100% susceptible to antibiotics such as ceftriaxone, penicillin, and vancomycin, 93% were sensitive to chloramphenicol, 83% to erythromycin, and only 13% to tetracycline.

Conclusion. Our data showed a high frequency of GBS infection in pregnant women and their newborns. A mandatory screening test and preventative medicine should be adopted to minimize the potentially fatal repercussions of this sickness.

Keywords: *Streptococcus agalactiae group B, molecular detection, pregnant women colonization, neonatal colonization, antimicrobial susceptibility*

Ethics approval. Prior to participation, all pregnant women signed a consent form detailing the study's objectives and their willingness to participate. The research protocol was approved by the Ethics Committee of the Babylon Technical Institute, Al Furat Al-Awsat Technical University (protocol No. 8643/27/7, September 24, 2023).

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Оригинальное исследование
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Выявление *Streptococcus agalactiae* молекулярными методами у беременных женщин и частота вертикальной передачи новорождённым в провинции Вавилон

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Аннотация

Введение. *Streptococcus agalactiae* — грамположительные, неподвижные и инкапсулированные кокки. На кровяном агаре они образуют узкую зону бета-гемолиза. Этот возбудитель при передаче от инфицированных матерей вызывает инвазивные бактериальные заболевания у новорождённых, в том числе сепсис, менингит, септицемию и пневмонию. *Streptococcus agalactiae* является патогеном, вызывающим первоочередную озабоченность общественного здравоохранения.

Цель работы — провести объективное исследование по выделению и молекулярному выявлению гена вирулентности стрептококка группы В (СГВ) и оценить частоту передачи инфекции от матери новорождённому.

Материалы и методы. В проспективное когортное исследование вошли 300 беременных женщин со сроком беременности более 35 нед. У всех участниц исследования собирали вагинальные мазки, всех женщин с диагностированным СГВ обследовали после родов, чтобы взять мазки у их новорождённых. Для оценки выделенных бактерий использовали традиционные микробиологические и молекулярные подходы.

Результаты. В исследовании приняли участие 60 (20%) из 300 беременных женщин и 16 (26,6%) их новорождённых. СГВ был обнаружен с помощью культуральных методов и подтверждён с помощью ПЦР с праймерами для выявления гена *atr* (гена домашнего хозяйства). Положительные изоляты были на 100% чувствительны к антибиотикам, таким как цефтриаксон, пенициллин и ванкомицин, 93% были чувствительны к хлорамфениколу, 83% — к эритромицину, 13% — к тетрациклину.

Заключение. Наши данные показали высокую частоту инфицирования СГВ у беременных женщин и их новорождённых. Необходимо проводить обязательный скрининг и профилактическое лечение, чтобы свести к минимуму потенциально смертельные последствия этого заболевания.

Ключевые слова: *стрептококки группы В, молекулярное обнаружение, колонизация беременных женщин, колонизация новорождённых, чувствительность к противомикробным препаратам*

Этическое утверждение. Перед участием все беременные женщины добровольно подписывали форму информированного согласия. Протокол исследования одобрен Этическим комитетом Вавилонского технического института Технического университета Аль-Фурат Аль-Аусат (протокол № 8643/27/7 от 24.09.2023).

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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

Group B *Streptococcus agalactiae* (GBS) are normal inhabitants of the human gastrointestinal and genitourinary systems. They are the primary cause of serious bacterial infections like neonatal meningitis, symptoms-free bacteriuria, Urinary tract infections (UTIs), bladder infection, inflammation of kidneys and pelvis, intra-amniotic infection (IAI), postpartum endometritis and pre- and postpartum bacteraemia. It is a gram-positive, opportunistic, beta-haemolytic bacteria with possible complications. It also causes infections of surgical wounds in pregnant women [1, 2]. By maternal rectovaginal colonization, GBS causes a variety of prenatal and maternal illnesses, consisting of infections in the mother, stillbirths, premature births, as well as early and late-onset sepsis in infants [3–6]. GBS-colonized mothers run the risk of vertical transmission of these bacteria to the newborn. It is one of the risk factors for early-onset sepsis in newborns [7]. This dynamic colonization constitutes the highest infectious disease risk for newborns. Notably, reports from international literature show that the rates of maternal GBS colonization were 6.5–36% in Europe [5, 6], 10–30% in North America [7, 8], 16.5–31.6% in African nations [9], and 1.4–36.7% in South America, which includes Brazil [10, 11], Chile[1], Peru [12], and Argentina [13].

The first recommendations for women's intrapartum antibiotic treatment to prevent GBS infection were established in 1966 [12]. Following the introduction of these treatments, the incidence of newborn GBS disease was reduced by 80% in America [14]. According to recommendations from the CDC (Centres for Disease Control and Prevention)[2], GBS can be diagnosed at 35–37 weeks of gestation using selective enrichment broth culture; however, this is not always possible in poor-income regions [5].

Maternal colonization with GBS is the greatest significant threat for newborn early-onset disease (EOD)

[1] WHO. Number of infant deaths (between birth and 11 months); 2022. URL: https://who.int/data/gho/data/indicators/indicator-details/GHO/number-of-infant-deaths

[2] Verani J.R., McGee L., Schrag S.J. Prevention of perinatal group B streptococcal disease: revised guidelines from CDC; 2010. URL: https://cdc.gov/Mmwr/preview/mmwrhtml/rr5910a1.htm

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(from 0 to 6 days) [15]. In 1973, a single infant was born to a GBS-infected woman from a group of 46 pregnant mothers, and EOD was first recorded. Among the neonates born to women with GBS infection, 2.17% were at risk for EOD at the time intrapartum antibiotic prophylaxis (IAP) has been suggested for both microbiological and risk-based screening in the United States [8]. According to the data from the World Health Organization (WHO), there were 1601 new-born fatalities in Sri Lanka in 2017, of which 0.04% were related to sepsis and other infectious diseases [11]. The convenient use of antibiotics and the detection of pathogens can further lower mortality rates in this population [15]. Consequently, monitoring maternal pathogen colonization is a crucial safeguard against infant infection. In 1980, the global prevalence of maternal GBS colonization was 18%, the Caribbean had a higher prevalence (34%), while Melanesia had the lowest (2%). Similar colonization rates (23%) were observed in North America, Europe, and Australia; Comparatively, the incidence was slightly higher in South Africa than in the Western nations, although it was lower in East Asia (9%), South and Southeast of Asia together was observed around (14%), West Africa (13%), and Central America (10%) [10]. The lack of publications in East Asia, South Asia, Southeast Asia, Western Africa and Central America may be the cause of the subpar prevalence estimates from these locations [10].

Polymerase chain reaction (PCR) assays provide an additional option for the quick identification of GBS colonization [11]. The **aim** of our study was the isolation of GBS strains and detection of the virulence gene by molecular method from pregnant mothers and their neonates, evaluation of the ratio of vertical transmission from infected women to their neonates, and reduction of the mortality and morbidity rates associated with GBS infection by using appropriate prophylactic antibiotics in Iraq, where neither screening for GBS nor an IAP protocol existed until the beginning of this study.

Materials and methods

Study Design

A prospective cohort study was designed that involved 300 pregnant women who were at 35 weeks or more of gestation. Three hundred vaginal swabs were taken before labor from all expectant mothers by the gynaecologist and 60 swabs from neonates born to GBS-positive women were taken shortly (to avoid contamination from other sources) after birth in the delivery room, including neonatal swabs from three sites (oral cavity, ear, and umbilicus). These neonates were born healthy.

Inclusion and Exclusion Criteria

Pregnant women who were in the final weeks of gestation (≥ 35 weeks) and were attending the Al-Zahraa Hospital of Obstetrics in Babylon (Iraq) were included in this study. This study excluded pregnant women who took antibiotics within 10 days of delivery and those who underwent caesarean surgery.

Bacteriological Identification of Isolates

All swab isolates from pregnant women and their neonates were screened for the period from November 2021 to June 2022. It was done by incubating specimens directly in the Todd-Hewitt Broth selective media overnight at 37ºC and subsequently subculturing them on blood agar to select the proper colony. The colonies were inspected and identified using the following criteria: a narrow beta haemolysis zone, gram positive cocci, bacitracin resistance, catalase negativity, sodium hippurate hydrolysis positivity, and CAMP positivity, in order to determine whether or not the plates contained GBS organisms.

Molecular identification of isolates

Samples that were positive for GBS in the culture method were sent for molecular detection after DNA extraction using the bacterial DNA extraction kit Geneaid (Taiwan). The primers and PCR steps used in the experiment to amplify *atr* housekeeping gene are listed in **Table 1** and **Table 2**. 1 µl of each upstream and downstream primer, 16 µl of nuclease-free water and 2 µl of extracted DNA were added to Master Mix to total volume of 25 µl in each reaction. Electrophoresis in a 2% agarose gel was used for detection of the PCR amplification product.

Antibiotic susceptibility test

The CLSI-recommended Kirby–Bauer disc diffusion technique with modifications on Muller Hinton agar and 5% blood was used to conduct the antibiotic sensitivity test [12]. In this method, antibiotics such as ciprofloxacin, ampicillin, penicillin, chloramphenicol,

Table 1. Primer sequence for *atr* genes [16]

Gene	Primer Sequence (5'–3')	Size, bp
Atr	F-5'CAACGATTCTCTCAGCTTTGTTAA3' R-5'TAAGAAATCTCTTGTGCGGATTTC3'	780

Table 2. PCR programme

Steps	Temperature, °C	Time, min	Cycles
Initial denaturation	94	1.00	1
Denaturation	94	1.00	
Annealing	55	0.75	30
Elongation	72	1.00	
Final extension	72	10.00	1
Hold	4	7.00	1

erythromycin, levofloxacin, vancomycin, tetracycline and levofloxacin were used.

Statistical Analysis

Student t-test was employed for quantitative variables and Chi-squared test was performed whenever available for binomial variables. *P* values less than 0.05 were considered to be statistically significant.

Results

The GBS colonization rate was 20% (60/300) among three hundred pregnant women and 26.6% (16/60) in neonates born of GBS-positive mothers (**Table 3**). In this study, the youngest woman was 15 years old, and the oldest woman was 44 years old, with the average age of participants being 28 years. The various sociodemographic variables are shown in **Table 4** and **Table 5**. On comparing the variations in GBS colonization rates between rural and urban residents, a statistically significant difference was found for GBS colonization among GBS-positive women (78.3% in rural residents versus 21.7% in urban residents; $p = 0.015$).

Table 3. GBS Isolates from Women and Their Newborns

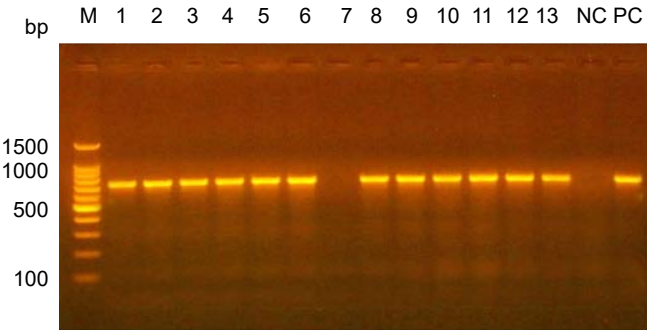
Isolates	Frequency	Percentage	Total
Pregnant women	60	20.0	300
Newborns	16	26.6	60

Molecular detection of all GBS isolates

Conventional PCR was used to detect a specific gene (*atr*) to confirm the bacteriological identification of *Streptococcus agalactiae*. This gene had a molecular size of 780 bp in gel electrophoresis as shown in **Figure**.

Antimicrobial sensitivity

The susceptibility of the isolates towards antibiotics is shown in **Table 6**. Based on the results of antimicrobial sensitivity, it was found that there is more than one antibiotic capable of inhibiting the growth of this bacteria, so they can be used as alternatives in the event of a drug allergy in the patient.



Electrophoresis of PCR product for *atr* gene on agarose gel. Lane M represents 100 bp DNA ladder, every lane (1–13) except lane 7 represented positive results. Lanes NC and PC represented negative and positive controls respectively.

Table 4. Age, residence and parity of patients

Variables		Culture result		Total	*p*
		negative (*n* = 240)	positive (*n* = 60)		
Age (mean ± SD), years Range		28.44 ± 8.20 (15–44)	29.92 ± 8.60 (15–41)	28.60 ± 8.20 (15–44)	0.3
Residence	Urban *n* (%)	92 (28.4)	13 (21.7)	105 (100.0)	0.015*
	Rural *n* (%)	148 (61.6)	47 (78.3)	195 (100.0)	
Parity	1 *n* (%)	26 (10.8)	7 (11.6)	33 (100.0)	0.2856
	2 *n* (%)	64 (26.6)	15 (25.0)	79 (100.0)	
	> 3 *n* (%)	150 (61.6)	38 (63.3)	188 (100.0)	

Note. *Represent a significant difference at $p \leq 0.05$.

Table 5. Age groups of female participants

Age group, years	Culture		Total	*p*
	negative, *n* (%)	positive, *n* (%)		
15–24	68 (28.4)	15 (20.0)	83 (27.7)	0.784
25–34	148 (61.6)	37 (61.1)	185 (61.6)	
35–44	24 (10.0)	8 (18.9)	32 (10.7)	
Total	240 (100.0)	60 (100.0)	300 (100.0)	

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Table 6. Antimicrobial sensitivity of maternal and neonate isolates

Antimicrobial agent	Sensitivity of GBS Isolates	
	mothers (*n* = 60), *n* (%)	neonates (*n* = 16), *n* (%)
Penicillin	60 (100.0)	16 (100.0)
Ampicillin	60 (100.0)	16 (100.0)
Levofloxacin	55 (91.6)	15 (93.7)
Vancomycin	60 (100.0)	16 (100.0)
Tetracycline	8 (13.3)	4 (25.0)
Ceftriaxone	60 (100.0)	16 (100.0)
Erythromycin	50 (83.3)	13 (81.2)
Chloramphenicol	56 (93.3)	15 (93.7)
Ciprofloxacin	58 (96.6)	16 (100.0)

Discussion

Due to the possibility of transmission from the mother to the fetus throughout the pregnancy and the postpartum period, resulting in serious illness or death, research into maternal GBS colonization is crucial. The effect of GBS infection is not restricted to childhood only but may continue to adulthood and may lead to dangerous neurological disorders. The prevalence of GBS maternal colonization worldwide is variable because this prevalence depends on many variable factors like hygienic conditions, socio demographic conditions, sample population, diagnostic techniques, and others. Our research is a conformational vertical transmission study conducted in Babylon (Iraq). This is also the first GBS cohort study that has been done in Babylon. 20% of pre-delivery mothers and 26.6% of neonates born of GBS-positive mothers were found to be colonized with GBS. The colonization rate in mothers observed in our study was comparable to the global data [8, 11].

The percentage of vertical transmission of GBS from infected mothers to neonates in the present study (26.6%) falls within the global report ranges. Compared to other global publications, however, this fraction of vertical transmission is lower than in studies conducted in Kuwait (35.5%), Bangladesh (38.0%) [17], China (7.6–16.7%) [19–21], the United States (53.8%) [22], and Eastern Ethiopia (53.8%) [23]. Other studies, such as those conducted by A. Joachim *et al*. in 2009 in Dar es Salaam, Tanzania (8.9%) [24] and by M. Gizachew *et al.* in Northwest Ethiopia was 10.4% [25], have shown results lower than that in the current study. Our explanation for this variation can be attributed to many reasons such as sample size, techniques used in diagnosis, period of stay in the delivery canal, premature rupture of membrane, and prophylaxis treatment by mothers.

The high vertical transmission rate of GBS contributes to substantial newborn and maternal morbidity and fatality. Vertical transmission of GBS can be prevented, hence healthcare practitioners and government officials must take this into account when developing initiatives to reduce maternal and newborn mortality [26]. Vertical transmission of GBS from a colonized woman to her neonate has not been explored properly, particularly in low income countries. A study investigating the risk variables that could be linked to vertical transmission would therefore help with the formulation of prevention measures. In the present study, we found that three maternal risk factors, mother work and antenatal care follow-up, were strongly associated with the vertical transmission of GBS from asymptomatic colonized mothers to their newborns. Women who had 4–5 ANC visits throughout their current pregnancy had a 20.9% lower risk of vertical GBS transmission to their neonates [17]. According to several studies, GBS colonization in pregnancy may be linked to a variety of factors including education, parity, mother's age, status of marriage, occupation, and an elevated body mass index [27, 28]. A substantial threat for morbidity and death rate in neonates with early-onset GBS illness has been linked to the colonization of the mother's birth canal.

This study showed statistically significant differences between GBS colonization in women in rural areas compared to those in urban areas (78.3% for rural versus 21.7% for urban; $p = 0.015$). This data disagrees with a study conducted in China by S. Li *et al.* in 2018, which showed no significant difference between women residing in rural and urban areas [29]. The plausible explanation for this difference is a lot of variables that make it easier for GBS to spread in rural regions such as low educational status, lack of health care facilities, and contamination from water and other sources. Furthermore, in this study, the neonatal samples were taken from three sites to increase the chance of GBS detection. The sample locations may influence vertical transmission rates. According to a study conducted in Pakistan [30], the risk of acquiring newborn GBS infection was much higher in sites like abdominal skin (53%) than in ear canals (18%). Furthermore, two Turkish studies published in the same year found that the rate of vertical transmission was 54.2% for three sites (throat, ear canal, and umbilicus) and 15.2% for two sites (throat and umbilicus) [31, 32]. In terms of parity, the prevalence of GBS colonization in our study was greater in more than three parities; the colonization rate of GBS in relation to parity was the highest during the reproductive years [33, 34]. The actual causes of such variable colonization are unknown and require additional research [35]. Thus, further research should be conducted to analyze the association of parity of women with GBS colonization.

The majority (61.6%) of participants in this study were between the ages of 25 and 34 years and had been pregnant at least once. This data differed from a similar study conducted by C. Turner et al. in 2012 on a population of refugees along the Thailand-Myanmar border in Southeast Asia, in which, most of the carriers were

in their 20s [36]. These outcomes are similar to those of C. Adware *et al.* from Cameroon in 2008 [37], P. Foumane *et al.* in 2002 in Cameroon [38], A. Mengist *et al.* in 2016 in Ethiopia [39], and N.M. Nkembe *et al.* in 2018 [40], who reported the values to be 75%, 60%, 64%, and 65%, respectively. Our explanations for this age range consist of two reasons, firstly, some women delay pregnancy due to the presence of health issues that prevent pregnancy at the start of a marriage, and secondly, some married couples delay childbearing in the initial years of marriage. In particular, each and every GBS strain was susceptible to penicillin, ampicillin, vancomycin, and ceftriaxone indicating that these antibiotics could be used for preventative purposes. The majority of isolates obtained from mothers were sensitive to ciprofloxacin (96.6%), chloramphenicol (93.3%), levofloxacin (91.6%), and erythromycin (83.3%). In 87% (50/60) of isolates, tetracycline resistance was observed. In other nations such as Tunisia (97.3%) [41] and Iran (96%) [42], tetracycline resistance was extremely prevalent. Its use is currently restricted since the emergence of resistance appears to be linked to the extensive administration of antibiotics [43] and efficient plasmid transfer [43]. Penicillin is the drug of choice for treatment. In the case of penicillin allergy and anaphylaxis, ampicillin or vancomycin might be used as an alternative for treatment.

Nonetheless, the use of PCR to identify GBS and other pathogenic bacteria is crucial. This technique is considered a crucial method in the medical field, it is utilized in a number of medical fields to identify clinical diseases [44–48] and other dangerous genetic diseases such as cancers [49–64].

Conclusion

According to our findings which have demonstrated a high frequency of GBS infection in pregnant women and their newborns, a mandatory screening test for all pregnant women should be implemented, as well as preventive medication should be provided, to avoid the potentially fatal effects of this illness.

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Analysis of antibiotic sensitivity of clinical strains of microorganisms with the Russian Mueller–Hinton broth

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Abstract

Introduction. One of the reasons for spreading antibiotic-resistant microorganisms is the uncontrolled use and inadequate empirical prescription of antibiotics which is not based on the results of the pathogen sensitivity testing. The broth dilution method and one of its implementation options, the reference microdilution method, in contrast to the disk diffusion method, allows testing virtually all pathogen-antibiotic combinations. To realize the method, a production technology of Russian Mueller–Hinton broth (MHB-Obolensk) has been developed under the import substitution program.

The aim. To evaluate the quality of the developed domestic Mueller–Hinton broth in comparative tests with its imported analog BD BBL (MHB-BD) in testing clinical strains of microorganisms, including microorganism–antibiotic combinations pairs which cannot be reliably investigated by the disc diffusion method.

Materials and methods. The study investigated the sensitivity of 47 clinical strains of Gram-positive and Gram-negative bacteria to antibiotics of various functional groups using the broth microdilution method with MHB-Obolensk and MHB-BD.

Results. The MICs values of antibiotics for clinical strains obtained with the developed and control media did not practically differ from each other or differed by +/– one dilution. The difference by two two-fold dilutions was noted when testing *Enterococcus faecium*–ampicillin, *Klebsiella pneumoniae*–meropenem, *Pseudomonas aeruginosa*–levofloxacin and *Staphylococcus aureus*–ciprofloxacin combinations. For the first two combinations, the MIC values were lower in MHB-Obolensk, and for the last two, they were higher than in MHB-BD. The differences obtained did not affect the clinical categories of sensitivity.

Conclusion. The antibiograms of clinical strains in developed Russian Mueller–Hinton broth was obtained, which did not differ from those for the comparison medium. MHB-Obolensk complies with the requirements of national and international standards and can be used to reliably test, among other things, current combinations of microorganism–antibiotic pairs that cannot be studied using the disk diffusion method.

Keywords: *Mueller–Hinton broth, import substitution program, broth microdilution method.*

Ethics approval. Only museum strains of microorganisms were used in the study; therefore, no biomedical ethics committee opinion or other documents are required to be submitted.

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Оригинальное исследование
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Использование отечественного бульона Мюллера–Хинтон для исследования антибиотикочувствительности клинических штаммов микроорганизмов

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Аннотация

Введение. Одна из причин распространения микроорганизмов, устойчивых к антимикробным препаратам (АМП), связана с бесконтрольным употреблением и неадекватным эмпирическим назначением антибиотиков, не основанным на результатах определения чувствительности возбудителя к ним. Метод разведений в бульоне и один из вариантов его исполнения — референтный метод микроразведений, в отличие от диско-диффузионного метода, позволяет тестировать практически все комбинации патоген–антибиотик. Для выполнения метода в рамках программы импортозамещения разработана технология производства отечественного бульона Мюллера–Хинтон (МХБ-Оболенск).

Цель исследования — оценить качество разработанного отечественного бульона МХБ-Оболенск в сравнительных испытаниях с импортным аналогом МХБ-BD («BD BBL») при тестировании клинических штаммов микроорганизмов, включая комбинации микроорганизм–АМП, которые нельзя достоверно исследовать диско-диффузионным методом.

Материалы и методы. В работе исследовали чувствительность 47 клинических штаммов грамположительных и грамотрицательных бактерий к АМП различных функциональных групп методом микроразведений в бульонах МХБ-Оболенск и МХБ-BD.

Результаты. Значения минимальных подавляющих концентраций (МПК) антибиотиков для клинических штаммов, полученные на разработанной и контрольной средах, между собой практически не отличались или отличались на +/– 1 разведение. Отличие на 2 двукратных разведения отмечено при тестировании комбинаций *Enterococcus faecium*–ампициллин, *Klebsiella pneumoniae*–меропенем, *Pseudomonas aeruginosa*–левофлоксацин и *Staphylococcus aureus*–ципрофлоксацин. Для двух первых комбинаций значения МПК на МХБ-Оболенск были ниже, а для двух последних — выше, чем на МХБ-BD. Полученные различия не отразились на клинических категориях чувствительности.

Заключение. На разработанном отечественном бульоне МХБ-Оболенск получены антибиотикограммы для клинических штаммов микроорганизмов, которые не отличались от их антибиотикограмм на контрольной среде. МХБ-Оболенск соответствует требованиям национальных и международных стандартов и с помощью него можно достоверно тестировать в том числе актуальные комбинации пар микроорганизм–АМП, которые нельзя исследовать диско-диффузионным методом.

Ключевые слова: *бульон Мюллера–Хинтон, импортозамещение, метод микроразведений в бульоне*

Этическое утверждение. В исследовании использованы только музейные штаммы микроорганизмов, поэтому не требуется представления заключения комитета по биомедицинской этике или иных документов.

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

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

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Introduction

The large-scale spread of bacteria resistant to various groups of antibiotics continues to be a public health problem worldwide [1]. The largest number of resistance cases is among healthcare-associated infections, including *Acinetobacter baumannii,* members of the *Enterobacterales* family*, Enterococcus faecium, Staphylococcus aureus*, *Pseudomonas aeruginosa*, etc. [2]. According to the estimates of experts, in 2019 alone, about 5 million cases of deaths caused by antibiotic-resistant bacteria have been identified worldwide [3], including multidrug-resistant or rifampicin-resistant tuberculosis[1].

The COVID-19 pandemic has exacerbated the existing global burden of antibiotic resistance mainly due to inappropriate and overuse of antibiotics [5].

The situation of the growing threat of antibiotic resistance is complicated by the significant decline in the development of new antimicrobials due to the lengthy procedure from development to implementation, high cost and low cost recovery. Currently, it takes about 10–15 years to advance a candidate antibiotic from preclinical to clinical trial stage [6]. Considering the critical necessity for new antibiotics, in 2017, the World Health Organization (WHO) published a list of resistant bacteria posing the greatest risk to human life and health[2], an updated list was released in 2024[3]. The updated list excludes 5 pathogen-antibiotic combinations (clarithromycin-resistant *Helicobacter pylori*; fluoroquinolone-resistant *Campylobacter* spp.; penicillin-resistant *Streptococcus pneumoniae*; third-generation cephalosporin-resistant *Providencia* spp.; vancomycin-resistant *S. aureus*) that were contained in the 2017 list, and added 4 new bacteria-antibiotic combinations: macrolide-resistant *Streptococcus group* A; penicillin-resistant *Streptococcus group* B; macrolide-resistant *S. pneumoniae*; rifampicin-resistant *Mycobacterium tuberculosis*. Carbapenem-resistant *P. aeruginosa* moved from the critical priority level to the high priority level due to reports of a decline in its global resistance to antibacterial drugs.

Another reason for the emergence of antimicrobial-resistant microorganisms is associated with uncontrolled and unjustified use of antibiotics, as well as inadequate empirical prescription of antibiotics without taking into account the results of sensitivity testing. Currently, the most common method of determining the sensitivity of microorganisms remains the disk-diffusion method. It is easy to perform and does not require expensive equipment. However, certain microorganism-antimicrobial combinations cannot be reliably tested by this method, which may lead to incorrect prescription of treatment regimens and further aggravate the situation with the spread of antibiotic resistance.

The broth dilution method and especially one of its variants, the microdilution method, which is recognized as a reference method, are free of such limitations[4]. This is a quantitative method, the use of which allows to determine the values of minimum inhibitory concentrations (MIC) of antimicrobials, which most accurately reflect the antimicrobial effect *in vitro* and are necessary to optimize the dosing regimen of antimicrobials.

The method allows testing such microorganism–antibiotic combinations, which cannot be reliably tested by disk-diffusion method, and some of which are included in the WHO list: *Salmonella* spp., resistant to ciprofloxacin; *Neisseria gonorrhoeae* resistant to cephalosporins and fluoroquinolones; *S. pneumoniae* and group A streptococci resistant to macrolides (azithromycin, clarithromycin and roxithromycin in case of resistance to erythromycin); non-brutonotyphoidal salmonellae resistant to fluoroquinolones (ciprofloxacin); etc.

It is recommended to use Mueller–Hinton broth (MHB) standardized for the content of divalent metal ions, thymidine and pH value because of their influence on the activity of some antibiotics. Until recently there was no industrial production of MHB in Russia, and the current situation with the imposition of economic sanctions against our country has led to the restriction of export of products for microbiological research. In this connection in the State Scientific Center of Applied Microbiology and Biotechnology, the production technology was developed and industrial production of MHB was established (RU RZN No. 2023/21584 from 29.11.2023). The broth has been tested on an expanded set of test strains and antimicrobials, and this study is devoted to studying the possibility of its use in testing clinical strains of microorganisms.

The aim of the study is to evaluate the quality of the developed domestic MHB in comparative tests with its imported analog BD BBL (MHB-BD) in testing clinical strains of microorganisms, including microorganism–antibiotic combinations pairs which cannot be reliably investigated by the disc diffusion method.

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[1] Tuberculosis: Multidrug-resistant (MDR-TB) or rifampicin-resistant TB (RR-TB). 2024. URL: https://www.who.int/news-room/questions-and-answers/item/tuberculosis-multidrug-resistant-tuberculosis-(mdr-tb)

[2] WHO publishes list of bacteria for which new antibiotics are urgently needed. 2017. URL: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

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

[4] GOST R ISO 20776-1. Infectious agent susceptibility testing and evaluation of the functional performance of articles for antimicrobial susceptibility testing. Part 1. Reference broth microdilution method for laboratory testing of the activity of antimicrobial agents against fast-growing aerobic bacteria causing infectious diseases. Moscow; 2022. 24 p.

Materials and methods

Nutrient media

MHB produced by the State Research Center for Applied Microbiology and Biotechnology (MHB-Obolensk; cat. no. O-282-K-1) and MHB produced by BD BBL (MHB-BD; cat. no. 212322) were used in the study, the latter was used as a control medium. For testing fastidious microorganisms, 5% lysed horse blood and 20 mg/L β-NAD (Sigma-Aldrich, cat. no. N8535) were added to the broths. Lysed horse blood was prepared from defibrinated horse blood (Ecolab) by adding sterile deionized water in a 1:1 ratio to defibrinated horse blood, placing it in a freezer for 8 ± 1 h at –20ºC. The thawed blood was then re-frozen and thawed once more at room temperature, repeating this cycle 4 times until complete lysis of blood cells. The lysed horse blood was then clarified by centrifugation at 7000 r/s for 30 min on an Eppendorf Centrifuge 5702 machine.

Microorganism strains under study

The strains of microorganisms deposited in the State Collection of Pathogenic Microorganisms (SCPM-Obolensk) were tested:

- 44 clinical strains of microorganisms previously isolated from patients treated at the inpatient department of the Regional Infectious Diseases Clinical Hospital of Yaroslavl Region and deposited in the SCPM-Obolensk: 14 strains of *K. pneumoniae*, 8 strains of *P. aeruginosa*, 4 strains of *A. baumannii*, 7 strains of *Staphylococcus* spp. (*S. aureus* — 6, *S. epidermidis* — 1), 7 strains of *Enterococcus* spp. (*E. faecium* — 4, *E . epidermidis* — 1), 7 strains of *Enterococcus* spp. (*E. faecium* — 4, *E. faecalis* — 1*, E. casseliflavus* — 1, *E. gallinarum* — 1), 2 strains of *Escherichia coli*, 1 strain of *Corynebacterium pseudodiphtheriticum*, 1 strain of *Morganella morganii*;
- 3 Campylobacter strains isolated from bird droppings of a farm in Moscow region and deposited in the State Research Center for Applied Biotechnology and Microbiology, Obolensk (*C. jejuni* — 2, *C. coli* — 1);
- 5 test strains used for routine quality control of testing and broths investigated in the work: *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *C. jejuni* ATCC 33560.

Antimicrobials

The following drugs were used in the study: amikacin (cat. no. A1774), ampicillin (cat. no. A9393), vancomycin (cat. no. 94747), gentamicin (cat. no. G3632), imipenem (cat. no. I0160), colistin (cat. no. C4461), levofloxacin (cat. no. 28266), linezolid (cat. no. PHR1885), meropenem (cat. no. PHR1772), tetracycline (cat. no. T8032), tigecycline (cat. no. PZ0021), trimethoprim (cat. no. T7883), ceftazidime (cat. no. PHR1847), ciprofloxacin (cat. no. 17850), erythromycin (cat. no. E6376), sulfamethoxazole (cat. no. S7507) — all manufactured by Sigma-Aldrich.

Broth microdilution method

The method was performed using a 96-well plate in accordance with the requirements of GOST R ISO 20776-1[4], as well as current versions of EUCAST and Russian recommendations for determining the sensitivity of microorganisms to antimicrobials[5, 6]. The obtained MIC values were used to determine the sensitivity categories of strains: S (sensitive under standard dosing regimen), R (resistant), I (sensitive under increased antimicrobial exposure). Testing of all microorganism-antimicrobial combinations was performed in 3 repetitions.

Physicochemical indicators of nutrient media quality

Physicochemical parameters of broth quality (amine nitrogen content, chloride content in terms of NaCl and loss in weight during drying) were determined in accordance with Methodological Guidelines 4.2.2316-08[7]. The content of calcium (Ca^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}) and zinc (Zn^{2+}) ions was determined by atomic emission spectrometry with inductively coupled plasma on the iCAP-6500 Duo plasma spectrometer (Thermo Scientific) in accordance with the requirements of GOST R ISO 27085-2012[8].

Thymidine content was evaluated by indirect method by determining the MIC value of trimethoprim/sulfamethoxazole in the study of control strain *E. faecalis* ATCC 29212. Obtaining an MIC ≤ 0.5/9.5 mg/L indicated an acceptable thymidine concentration of less than 0.03 mg/L in the broth[9].

Statistical methods

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[5] European Committee for Antimicrobial Susceptibility Testing (EUCAST). URL: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/QC/v_14.0_EUCAST_QC_tables_routine_and_extended_QC.pdf

[6] Russian recommendations "Determination of susceptibility of microorganisms to antimicrobial agents" (version 2024-02). KMAX. 2024;26(2). URL: https://microbius.ru/library/rossiyskie-rekomendatsii-opredelenie-chuvstvitelnosti-mikroorganizmov-k-antimikrobnym-preparatam

[7] Methodological guidelines 4.2.2316-08. 4.2. Methods of control of bacteriological nutrient media: Methodological guidelines. Moscow; 2008.

[8] GOST R ISO 27085-2012. Animal feeds. Determination of calcium, sodium, phosphorus, magnesium, potassium, iron, zinc, copper, manganese, cobalt, molybdenum, arsenic, lead and cadmium by ICP-AES method. Moscow; 2014.

[9] GOST P 59786-2021/ISO/TS 16782:2016. Clinical laboratory tests. Acceptability criteria for lots of dehydrated agar and Mueller-Hinton broth used for antibiotic sensitivity assessment. Moscow; 2021. 30 p.

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The results were processed using the MS Excel program package. The reliability of different mean values was assessed using Student's t-criterion. Two-tailed Fisher's exact test was used in comparative analysis, significance level $p < 0.05$.

For test strains of microorganisms, the obtained values of MIC of antibiotics were compared with target values and acceptable ranges. The obtained results in accordance with GOST R ISO 20776-2-2010[10] were presented in the following evaluation categories:

- C — the mean value corresponds to the target value;
- H — high, the mean value is higher than the target value by 1 twofold dilution;
- L — low, the mean value is lower than the target value by 1 twofold dilution;
- VH — very high, the mean value is higher than the target value by 2 twofold dilutions, but is within the range of acceptable values;
- VL — very low, the mean value is lower than the target value by 2 twofold dilutions, but is within the range of acceptable values;
- LE — low error, the mean value is less than the lowest acceptable value;
- HE — high error, the mean value is more than the highest acceptable value.

Results

Before the beginning of the study, the quality control of MHB-Obolensk was carried out using control strains of *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *C. jejuni* ATCC 33560 and antibiotics, the results of sensitivity to which depend on the quality of the used MHB [9-11]. When selecting antibiotics, we proceeded from the following requirements of the standards: to obtain reliable results of sensitivity testing to tetracyclines, penicillins, aminoglycosides, macrolides and fluoroquinolones, it is recommended to use MHB with an optimal pH value of 7.2–7.4. For aminoglycosides, tetracyclines and fluoroquinolones the medium should be strictly balanced in terms of calcium and magnesium ions, for tigecycline and carbapenems – in terms of concentration of manganese and zinc ions, respectively, and for sulfonamide drugs the concentration of thymidine in the broth is critical.

During the quality control of MHB-Obolensk, the MIC values of the antibiotics obtained were classified as category C in 84.4% of cases. In 8.0% of cases the obtained MIC values of antibiotics were classified as category H, and in the remaining 7.6% of cases the obtained MIC values were qualified as L. No values categorized as VH, VL, LE and HE were obtained during the study. The MIC values of antibiotics for the test strains determined on the control medium MHB-BD were also within the permissible intervals. The results obtained testified to the high quality of the analyzed nutrient media and the possibility of their use for the study of clinical strains.

Upon further research on the developed and control broths, we studied the sensitivity of *Enterobacterales* representatives (*K. pneumoniae*, *E. coli* and *M. morganii*) to imipenem, meropenem, ceftazidime, levofloxacin, ciprofloxacin, ampicillin, colistin, gentamicin and trimethoprim/sulfamethoxazole, and *E. coli* — additionally to tigecycline, *P. aeruginosa* — to imipenem, meropenem, ceftazidime, levofloxacin, ciprofloxacin and colistin, *A. baumannii* — to imipenem, meropenem, levofloxacin, ciprofloxacin, colistin and gentamicin, *Campylobacter* spp. — to ciprofloxacin, tetracycline and erythromycin, *Staphylococcus* spp. — to levofloxacin, ciprofloxacin, linezolid, vancomycin, tetracycline, gentamicin, erythromycin, tigecycline and trimethoprim/sulfamethoxazole, *Enterococcus* spp. — to imipenem, levofloxacin, ciprofloxacin, linezolid, vancomycin, ampicillin and tigecycline, *C. pseudodiphtheriticum* — to ciprofloxacin, linezolid, vancomycin and tetracycline.

The MIC values of antibiotics obtained on MHB-Obolensk and control MHB-BD were almost identical to each other. When 8 antimicrobial-microorganism combinations were tested, differences in MIC per 1 two-fold dilution were noted. For 4 combinations on MHB-BD they exceeded the values on control broth and amounted to 0.12 mg/L vs. 0.06 mg/L for meropenem against *K. pneumoniae* 16, 0.06 mg/L vs. 0.03 mg/L for imipenem against *K. pneumoniae* 203, 0.25 mg/L versus 0.125 mg/L for ceftazidime against *E. coli* 1169/70, and 0.06 mg/L versus 0.03 mg/L for levofloxacin against *A. baumannii* 494. In contrast, they were lower for 4 combinations and were 0.03 mg/L versus 0.06 mL/L for levofloxacin on control broth against *E. faecalis* 2211406, 16.0 mg/L vs. 32 mg/L for tetracycline against *C. jejuni* F-2, 0.25 mg/L vs. 0.5 mg/L for colistin against *K. pneumoniae* 1643, 0.03 mg/L vs. 0.06 mg/L for vancomycin against *S. aureus* 2202263.

Differences between the two MHBs in MIC results at 2 two-fold dilutions were observed when 4 combinations were tested: *K. pneumoniae* 1142–meropenem, *P. aeruginosa* 265–levofloxacin, *S. aureus* 2202309–ciprofloxacin and *E. faecium* 613–ampicillin. At the same time, the MIC values of levofloxacin and ciprofloxacin, both equal to 0.12 mg/L, respectively, were higher on MHB-Obolensk than on control broth (0.03 and 0.03 mg/L), and the MIC values of meropenem and ampicillin, equal to 0.06 and 0.03 mg/L, respectively, were lower than on MHB-BD (0.016 and 0.008 mg/L).

However, the resulting differences in MIC val-

[10] GOST R ISO 20776-2-2010. Clinical laboratory tests and in vitro diagnostic test systems. Infectious agent susceptibility testing and evaluation of functional performance of products for antimicrobial susceptibility testing. Part 2. Evaluation of the functional performance of antimicrobial susceptibility testing products.

ues did not affect the evaluation of clinical sensitivity categories of the clinical strains tested. The results of antimicrobial sensitivity testing (in clinical sensitivity categories) for 44 clinical strains of microorganisms and 3 Campylobacter strains isolated from farm birds are presented in the **Table**.

All strains of *K. pneumoniae* studied in this work are mainly sensitive to the tested antibiotics under standard dosing regimen. One strain was resistant to ceftazidime and ciprofloxacin, 2 — to ampicillin, 3 — to gentamicin. Both *E. coli* strains were sensitive under standard dosing regimen to imipenem, meropenem, ceftazidime, levofloxacin, ampicillin, tigecycline and trimethoprim/sulfamethoxazole. One of them showed resistance to ciprofloxacin, colistin and gentamicin, while the other was sensitive to these antimicrobials. The *M. morganii* strain was interpreted as sensitive under standard dosing regimen to imipenem, meropenem, ceftazidime, levofloxacin, ciprofloxacin, ampicillin, colistin and trimethoprim/sulfamethoxazole, but resistant to gentamicin.

Antibiogram analysis of *P. aeruginosa* strains showed that all were sensitive to meropenem, and at increased antimicrobial exposure were also sensitive to imipenem, ceftazidime, levofloxacin, and ciprofloxacin. One of the 8 pseudomonad strains tested showed resistance to colistin, while the other 7 were sensitive to it.

A. baumannii strains were sensitive to imipenem, meropenem, levofloxacin, and colistin, and to ciprofloxacin at increased antimicrobial exposure. Only 1 strain was sensitive to gentamicin, and the others showed resistance, as did all 4 strains tested to trimethoprim/sulfamethoxazole.

One strain of Campylobacter showed resistance to ciprofloxacin, tetracycline and erythromycin, two others showed sensitivity to tetracycline and erythromycin and sensitivity, but with increased exposure, to ciprofloxacin.

All investigated gram-positive strains of *Staphylococcus* spp. and *Enterococcus* spp. are sensitive to linezolid. Against levofloxacin and ciprofloxacin, all *Staphylococcus* spp. strains were interpreted as sensitive at increased antimicrobial exposure, and against vancomycin and tigecycline as sensitive at standard dosing regimen. One of the 7 *Staphylococcus* spp. strains tested showed resistance to tetracycline and erythromycin, while the rest were sensitive under the standard dosing regimen. Regarding gentamicin and trimethoprim/sulfamethoxazole, only 4 strains of *Staphylococcus* spp. were sensitive to these antimicrobials, while the remaining 3 were resistant to them.

All *Enterococcus* spp. strains were sensitive to levofloxacin, ciprofloxacin and ampicillin at standard dosing regimen, and to imipenem — at increased exposure. At the same time, 3 strains of *E. faecium* showed resistance to vancomycin and tigecycline, while the other 4 strains showed sensitivity.

The *C. pseudodiphtheriticum* strain tested in combination with linezolid, vancomycin and tetracycline was interpreted as sensitive and in combination with ciprofloxacin as resistant. Using the control nutrient medium MHB-BD, similar antibiograms were obtained for all tested microorganism strains.

Discussion

In this study, the sensitivity of Gram-negative and Gram-positive bacteria (including fastidious bacteria) isolated from sick people and farm animals to antibiotics of different groups was tested on MHB-Obolensk. The list of antimicrobials included antibiotics, sensitivity to which cannot be determined by disk-diffusion method (ciprofloxacin only for salmonella, vancomycin and colistin for all microorganisms); antibiotics that act as quality markers for MHB (tetracycline, gentamicin, erythromycin, tigecycline, trimethoprim/sulfamethoxazole, levofloxacin, imipenem, meropenem, ampicillin) and others (linezolid, ceftazidime) [9–11].

At the State Research Center for Applied Microbiology and Biotechnology, we managed to design an MHB that meets the requirements of national and international standards (see footnotes 5, 8), based on a specially developed hydrochloric acid hydrolysate of modified casein. The pH value of the developed broth is in the range of 7.2–7.3, calcium ion content is in the range of 20.0–25.0 mg/L, magnesium — 10.0–12.0 mg/L, manganese level in it is less than 8.0 mg/L, zinc — less than 3.0 mg/L, thymidine — less than 0.03 mg/L. Other physicochemical quality parameters, the requirements for which are not regulated by the standard (see footnote 8), do not differ from those for the imported analog: the content of amine nitrogen varies from 4.7 to 5.0%, chlorides — from 27.5 to 28.7%, and the loss in weight during drying is 3.8–4.0%.

The use of MHB with such characteristics allowed to obtain the results of sensitivity categories of 47 clinical strains of microorganisms to 14 antibiotics, not differing from those on the control medium of a reliable manufacturer — MHB-BD.

The produced broth can be used for routine performance of serial dilutions method in macro- and micro versions, for commercial tests (in the format of tablets and MIC-strips with dried antibiotic substances), as well as for automatic analyzers.

Conclusion

The antibiograms of clinical strains in developed Russian Mueller–Hinton broth was obtained, which did not differ from those for the comparison medium. MHB-Obolensk complies with the requirements of national and international standards and can be used to reliably test, among other things, current combinations of microorganism–antibiotic pairs that cannot be studied using the disk diffusion method.

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Results of clinical strain testing by broth microdilution method using MHB-Obolensk and MHB-BD

Antibiotics	Nutrient media	*K. pneumoniae* — 14		*E. coli* — 2		*M. morganii* — 1		*P. aeruginosa* — 8		*A. baumannii* — 4		*Campylobacter* spp. — 3		*Staphylococcus* spp. — 7		*Enterococcus* spp. — 7		*C. pseudodiphtheriticum* — 1	
		n	SC	*n*	SC	*n*	SC	*n*	SC	*n*	SC	*n*	SC	*n*	SC	*n*	SC	*n*	SC
Imipenem	MHB-Obolensk	14	S	2	S	1	S	8	I	4	S	–	–	–	–	7	I	–	–
	MHB-BD	14	S	2	S	1	S	8	I	4	S	–	–	–	–	7	I	–	–
Meropenem	MHB-Obolensk	14	S	2	S	1	S	8	S	4	S	–	–	–	–	–	–	–	–
	MHB-BD	14	S	2	S	1	S	8	S	4	S	–	–	–	–	–	–	–	–
Ceftazidime	MHB-Obolensk	1 13	R S	2	S	1	S	8	I	–	–	–	–	–	–	–	–	–	–
	MHB-BD	1 13	R S	2	S	1	S	8	I	–	–	–	–	–	–	–	–	–	–
Levofloxacin	MHB-Obolensk	14	S	2	S	1	S	8	I	4	S	–	–	7	I	7	S	–	–
	MHB-BD	14	S	2	S	1	S	8	I	4	S	–	–	7	I	7	S	–	–
Ciprofloxacin	MHB-Obolensk	1 13	R S	1 1	R S	1	S	8	I	4	I	1 2	R I	7	I	7	S	1	R
	MHB-BD	1 13	R S	1 1	R S	1	S	8	I	4	I	1 2	R I	7	I	7	S	1	R
Linezolid	MHB-Obolensk	–	–	–	–	–	–	–	–	–	–	–	–	7	S	7	S	1	S
	MHB-BD	–	–	–	–	–	–	–	–	–	–	–	–	7	S	7	S	1	S
Vancomycin	MHB-Obolensk	–	–	–	–	–	–	–	–	–	–	–	–	7	S	3 4	R S	1	S
	MHB-BD	–	–	–	–	–	–	–	–	–	–	–	–	7	S	3 4	R S	1	S
Ampicillin	MHB-Obolensk	2 12	R S	2	S	1	S	–	–	–	–	–	–	–	–	7	S	–	–
	MHB-BD	2 12	R S	2	S	1	S	–	–	–	–	–	–	–	–	7	S	–	–
Colistin	MHB-Obolensk	14	S	1 1	R S	1	S	1 7	R S	4	S	–	–	–	–	–	–	–	–
	MHB-BD	14	S	1 1	R S	1	S	1 7	R S	4	S	–	–	–	–	–	–	–	–
Tetracycline	MHB-Obolensk	–	–	–	–	–	–	–	–	–	–	1 2	R S	1 6	R S	–	–	1	S
	MHB-BD	–	–	–	–	–	–	–	–	–	–	1 2	R S	1 6	R S	–	–	1	S
Gentamicin	MHB-Obolensk	3 11	R S	1 1	R S	1 1	R R	–	–	3 1	R S	–	–	3 4	R S	–	–	–	–
	MHB-BD	3 11	R S	1 1	R S	1 1	R R	–	–	3 1	R S	–	–	3 4	R S	–	–	–	–
Erythromycin	MHB-Obolensk	–	–	–	–	–	–	–	–	–	–	1 2	R S	1 6	R S	–	–	–	–
	MHB-BD	–	–	–	–	–	–	–	–	–	–	1 2	R S	1 6	R S	–	–	–	–
Tigecycline	MHB-Obolensk	–	–	2	S	–	–	–	–	–	–	–	–	7	S	3 4	R S	–	–
	MHB-BD	–	–	2	S	–	–	–	–	–	–	–	–	7	S	3 4	R S	–	–
Trimethoprim-sulfamethoxazole	MHB-Obolensk	14	S	2	S	1	S	–	–	4	R	–	–	3 4	R S	–	–	–	–
	MHB-BD	14	S	2	S	1	S	–	–	4	R	–	–	3 4	R S	–	–	–	–

Note. *n* — number of strains; SC — sensitivity category. A dash — testing for this antibiotic has not been performed; S — sensitive to standard dosing regimen; R — resistant; I — sensitive to increased exposure to antibiotic.

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