3
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16+

CONTENTS

ORIGINAL RESEARCHES

SCIENCE AND PRACTICE

REVIEWS

ANNIVERSARIES

OBITUARIES

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СОДЕРЖАНИЕ

ОРИГИНАЛЬНЫЕ ИССЛЕДОВАНИЯ

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ЮБИЛЕИ

НЕКРОЛОГИ

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ORIGINAL STUDY ARTICLES

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Data update on anthrax stationary hazardous areas and soil foci as a basis for improving epizootological and epidemiological monitoring for anthrax in the Russian Federation

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Abstract

Introduction. Anthrax is registered annually in the Russian Federation. The constant risk of complication of the epizootological and epidemiological situation on anthrax is due to the widespread distribution of soil foci of infection (anthrax burials (AB), «pestilence fields») and associated stationary hazardous areas (SHA).

The aim is to update data on anthrax SHA and soil foci in order to improve epidemiological surveillance of anthrax in the Russian Federation.

Materials and methods. Archival and reference materials on anthrax SHA and soil foci, accounting and reporting data of territorial bodies of Rospotrebnadzor and veterinary service were used.

The selection of criteria for characterizing anthrax SHA, AB and «pestilence fields» was carried out, using which the structure of databases of anthrax SHA and soil foci was developed.

Results. For the first time, electronic databases of anthrax SHA and soil foci on the territory of Russia were developed, containing updated information of the characteristics of 32566 SHA and 3314 soil foci (3185 AB and 129 «pestilence fields»). Analysis of the data revealed a decrease in the number of SHA and AB in the country compared to the reference data, as well as a lack of correlation between the counted number of SHA and AB in most regions, indicating the presence of a large number of unreported AB and the persistence of potential risks of infection situation complications.

Conclusion. The introduction of up-to-date databases of anthrax SHA and soil foci into the practice of Rospotrebnadzor bodies and institutions and veterinary services will improve the level of information support and efficiency of epidemiological surveillance of anthrax in the Russian Federation.

Keywords: *anthrax, stationary hazardous area, soil focus, anthrax burial, «pestilence field», epizootological and epidemiological monitoring.*

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Оригинальное исследование
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Актуализация данных о сибиреязвенных стационарно неблагополучных пунктах и почвенных очагах как основа совершенствования эпизоотолого-эпидемиологического мониторинга сибирской язвы в Российской Федерации

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Аннотация

Введение. Сибирская язва в Российской Федерации регистрируется ежегодно. Сохранение постоянного риска осложнения эпизоотолого-эпидемиологической ситуации по сибирской язве обусловлено повсеместным распространением почвенных очагов инфекции (сибиреязвенных захоронений (СЯЗ), «моровых полей») и связанных с ними стационарно неблагополучных пунктов (СНП).

Цель работы — актуализация данных о сибиреязвенных СНП и почвенных очагах с целью совершенствования эпидемиологического надзора за сибирской язвой в России.

Материалы и методы. Использованы архивные, справочные материалы о сибиреязвенных СНП и почвенных очагах, учётные и отчётные данные территориальных органов Роспотребнадзора и ветеринарной службы. Осуществлён подбор критериев характеристики сибиреязвенных СНП, СЯЗ и «моровых полей», с использованием которых разработана структура баз данных СНП и почвенных очагов сибирской язвы.

Результаты. Впервые разработаны электронные базы данных сибиреязвенных СНП и почвенных очагов на территории России, содержащие актуализированную информацию о характеристиках 32 566 СНП и 3314 почвенных очагов (3185 СЯЗ и 129 «моровых полей»). Анализ данных выявил снижение числа СНП и СЯЗ на территории страны по сравнению со справочными сведениями, а также отсутствие корреляции между учтённым количеством СНП и СЯЗ в большинстве регионов, что указывает на наличие большого числа неучтённых СЯЗ и сохранение потенциальных рисков осложнения ситуации по инфекции.

Заключение. Внедрение в практику органов и учреждений Роспотребнадзора, ветеринарной службы актуальных баз данных сибиреязвенных СНП и почвенных очагов позволит повысить уровень информационного обеспечения и эффективности эпидемиологического надзора за сибирской язвой в России.

Ключевые слова: *сибирская язва, стационарно неблагополучный по сибирской язве пункт, почвенный очаг, сибиреязвенное захоронение, «моровое поле», эпизоотолого-эпидемиологический мониторинг*

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

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Introduction

Anthrax is a highly dangerous zoonotic infectious disease characterized by its practically ubiquitous distribution in the Russian Federation [1, 2]. The persistence of epizootiological and epidemiological instability regarding anthrax is due to the existence of *Bacillus anthracis* in the form of spores resistant to environmental factors, capable of long-term survival in the soil and the formation of numerous soil foci—anthrax burials (AB), "pestilence fields (extensive areas without clear boundaries where mass animal deaths associated with anthrax epizootics were observed in the past), the indicators of which are stationary hazardous areas (SHA) [2–4].

In Russia, over 37,000 SHA active from 1900 to 2003 (several SHA since the late 19th century) have been registered more than 70,000 times [5], and at least 4,000 AB [6–10]. A significant number of hazardous areas and soil foci, along with incomplete surveillance and coverage of vaccination for livestock, as well as individuals at high risk of infection, maintain a potential threat of worsening the situation with zoonotic diseases in the country [11, 12].

The stabilization of the situation regarding anthrax infection can be facilitated by an approach to the comprehensive solution of tasks in epizootiological and epidemiological monitoring, the most important component of which is the updating (revision and clarification of data) and systematization of information about anthrax and soil foci in each subject of the Russian Federation. The generalization and analysis of updated information on SHA and soil foci will ensure the improvement of surveillance over anthrax, increasing the effectiveness of managerial decisions during disease outbreak investigations, including the use of modern geographic information systems in conducting a combined correlational analysis of the characteristics of anthrax activity depending on the influence of environmental factors (natural-geographical, social, etc.), preventing the emergence of new infection cases [1, 3, 13–16].

The aim of the study is to update data on SHA and anthrax soil foci (anthrax, "pestilence fields ") to improve epidemiological surveillance of anthrax in Russia.

Materials and methods

Archival and reference information on anthrax in SHA (Cadastre of stationary hazardous areas of anthrax in the Russian Federation, edited by B.L. Cherkassky, 2005, hereinafter referred to as the Cadastre) [5] and soil foci (List of animal burial sites (including anthrax sites) located in the territory of the Russian Federation, 2011–2013, in 5 parts, hereinafter referred to as the List) [6–10], as well as accounting and reporting data from the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor) by subjects of the Russian Federation and territorial veterinary service bodies, were used as research materials.

The necessity to update the Cadastre [5] is due to changes in the administrative status of a large number of settlements: renaming and re-subordination of municipal formations, the transfer of several settlements to other municipal formations (consolidation, etc.), the liquidation of many settlements under the status of "former settlement", etc., as well as the inclusion of data on the settlement activity in the 21st century. The importance of updating the List [6–10] in the regions of Russia is related to the elimination of a large number of AB, the identification of previously unreported burials, the clarification of AB characteristics: AB sizes, compliance of AB facilities with veterinary and sanitary regulations for maintenance, the presence of administrative and economic affiliation of AB, the sanitary protection zone (SPZ) of AB, geographical coordinates of location, etc.

With the aim of updating data on SHA and anthrax soil foci in the subjects of the Russian Federation, an expanded selection of criteria for the characteristics of SHA, AB and "pestilence fields " has been carried out, based on which the structure of regional databases for SHA and soil foci of infection has been developed.

Specialists from the Stavropol Anti-Plague Institute of Rospotrebnadzor, the Central Research Institute of Epidemiology of Rospotrebnadzor, the Irkutsk Anti-Plague Institute of Rospotrebnadzor and the Rostov State Medical University have conducted the systematization and analysis of the established regional databases of anthrax and soil foci for each subject, and created two generalized electronic databases (EDB): "Stationary Hazardous Areas of Anthrax on the Territory of the Russian Federation" and "Soil Foci of Anthrax on the Territory of the Russian Federation."

The database "Stationary Hazardous Areas of Anthrax on the Territory of the Russian Federation" is an MS Excel workbook with the following structure: summary data on SHA in the territory of Russia and information on the presence and characteristics of SHA in the subjects of the Russian Federation.

The SHA EDB for each subject has the following structure:

1) data on SHA in accordance with the Cadastre [5]: subject of the Russian Federation, administrative district, municipal formation, populated area;

2) updated data on SHA with an indication of the current status of SHA (existing, former/liquidated settlement, incorporated into another settlement, renamed, etc.): subject of the Russian Federation, administrative district, municipal formation, settlement;

3) information on SHA activity: years of SHA activity manifestations;

4) information on the morbidity of animals and humans;

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5) geographical coordinates of the SHA location.

The EDB "Soil Foci of Anthrax in the Territory of the Russian Federation" is a MS Excel workbook that includes summarized information about anthrax soil foci (AB, "pestilence fields ") and data on the presence and characteristics of anthrax soil foci in the subjects of Russia.

The EDB of soil foci for each subject has the following structure:

1) updated data on the AB ("pestilence fields "): subject of the Russian Federation, administrative district, municipal formation, populated area;

2) availability of a veterinary sanitary card for the breeding farm;

3) years of animal burial (for AB), animal mortality (for the "pestilence fields ");

4) type of soil focus: AB (earth pit, equipped animal burial ground, Bekari biothermal pit, wooden log cabin), "pestilence field ";

5) method of animal disposal: not disposed of (burial of animal carcasses), incineration (burial of ash residue);

6) the number of buried/deceased animals by species;

7) AB Square ("pestilence field ");

8) Veterinary and sanitary condition of the AB: fence, signboards, hill, moat;

9) presence of the AB in the flood zone: yes/no;

10) use of the AB: conserved/used;

11) administrative and economic affiliation of the AB: presence of a land user (custodian);

12) characteristics of the AB SPZ : information on the presence and size of the SPZ, economic use of the regulated SPZ (within a radius of 1000 m): presence of residential development, parks, recreational areas, livestock farms, cattle transit routes, planned construction sites, exploration and extraction of mineral resources, flood zones, etc.;

13) geographical coordinates of the location of the AB ("pestilence field").

Certificates of state registration have been obtained for the developed databases of SHA and soil foci in the subjects of the Russian Federation[1, 2].

Results

Results of the data update on anthrax in the subjects of the Russian Federation

The EDB "Stationary Hazardous Areas of Anthrax on the Territory of the Russian Federation" as of April 2025 contains updated information on the characteristics and locations of 32,566 SHA (**Table 1**).

In the subjects of the Russian Federation as a whole, 4795 fewer SHA have been updated compared to the Cadastre data [5]. The number of accounted SHA in most subjects of Russia in the Central, Northwestern, Volga and Ural federal districts is less than the number of SHA indicated in the Cadastre [5]. An increase in the number of SHA has been identified in several subjects of the Southern, North Caucasian, Siberian and Far Eastern federal districts.

In 18 subjects of the ***Central Federal District***, 7969 SHA have been updated. Compared to the Cadastre [5], the number of SHA decreased by 2458 points. The highest number of SHA (over 700 points) were recorded in the Oryol (759 SHA), Voronezh (781) and Ryazan (840) regions. From 2000 to 2024, 35 SHA were active in 7 subjects of the Central Federal District: in Voronezh (12 SHA), Kursk (10), Tambov (5), Belgorod (4), Tula (2), Oryol (1) and Ryazan (1) regions. Geographical coordinates of the locations have been established for 7810 (98%) SHA.

In the ***Northwestern Federal District***, out of 2148 SHA [5], information on 1500 SHA has been updated in 10 out of 11 subjects, and there are no registered SHA in the federal city of St. Petersburg. The majority of SHA are recorded in the Novgorod (772 SHA) and Vologda (435) regions, while the fewest are in the Nenets Autonomous District (16), Kaliningrad (3) and Murmansk (2) regions. Since 2000, anthrax infection has not been observed in the Northwestern Federal District. Geographical coordinates are known for 98.6% of SHA in the Northwestern Federal District.

In the ***Southern Federal District***, information has been updated on 2,589 SHA located in all subjects, except for the city of federal significance Sevastopol. The number of SHA exceeded the Cadastre data [5] by 136 points, which is primarily due to the inclusion of the Republic of Crimea in the Southern Federal District, where 211 SHA are accounted for, as well as the increase in the number of SHA in the Volgograd region (from 719 to 727), the Republic of Kalmykia (from 79 to 99), and the Republic of Adygea (from 74 to 75). The maximum number of SHA was recorded in the Rostov region — 797 points. In the Southern Federal District, manifestations of the infection were registered in 35 SHA from 2000 to 2016, with a relatively larger number in the Rostov region (11 SHA active in 2000, 2002, 2003, 2005–2007, 2010, 2014). Geographical coordinates have been determined for the majority of SHA (96.6%), and in the republics of Kalmykia (99 SHA) and Crimea (211), for all points.

In all 7 subjects of the ***North Caucasian Federal District***, 1256 SHA have been recorded, which is 31 points more than in the Cadastre [5]. Since 2000, the infection has been detected in 49 SHA across 5 regions, during this period, anthrax was only absent in the Kabardino-Balkar and Karachay-Cherkess republics.

[1] Database "Permanently affected by anthrax sites in the territory of the Russian Federation", certificate of state registration dated 08/01/2024 No. 2024623389.

[2] Database "Soil foci of anthrax on the territory of the Russian Federation", certificate of state registration dated 05.11.2024 No. 2024624926.

Table 1. Summary data on updated SHA in the Russian Federation

Federal District	Number of SHA according to the Cadastre [5]	Number of SHA according to the updated data	Number of SHA with manifestations of activity since 2000	Number of SHA with information about cases of disease in animals	Number of SHA with information about cases of disease in humans	Number of SHA with known geographical coordinates of the locations
Central Federal District	10,427	7969	35	1549	111	7810
Northwestern Federal District	2148	1500	–	63	9	1479
Southern Federal District	2453	2589	35	953	172	2500
North Caucasian Federal District	1225	1256	49	919	547	1234
Volga Federal District	13 113	10,947	32	4001	804	10,511
Ural Federal District	2096	1945	4	187	2	1896
Siberian Federal District	4766	4990	24	2942	168	3643
Far Eastern Federal District	1133	1370	5	466	118	1093
Total	37,361	32,566	184	11,080	1931	30,166

In the Republic of Dagestan, the predominant number of SHA in the North Caucasian Federal District was recorded — 516 (41%), of which 25 outbreaks of the disease were active in 23 SHA from 2000 to 2022. In the Stavropol Krai, out of 361 SHA from 2001 to 2022, 10 outbreaks were registered at 10 points. Overall, the geographical coordinates of the locations have been determined for more than 98% of SHA in the North Caucasian Federal District (in the Republic of Ingushetia and the Kabardino-Balkar Republic — for 100% of SHA).

In the ***Volga Federal District*** (a total of 14 subjects), the number of hazardous production facilities decreased by 2,166 — 10,947 SHA were updated. The reduction in the number of recorded SHA is most pronounced in the Kirov region (from 1275 to 534 SHA), the Republic of Bashkortostan (from 1587 to 1292), the Orenburg region (from 1064 to 822), and the Ulyanovsk region (from 608 to 372). More than 60% of all SHA in the Volga Federal District are localized in 5 subjects: Nizhny Novgorod Region (1893), the Republic of Tatarstan (1320), the Republic of Bashkortostan (1292), the Chuvash Republic — Chuvashia (1231), and Saratov Region (1040). At the same time, in the Republic of Tatarstan, the number of SHA exceeds the Cadastre data [5] by 111 points. Since 2000, anthrax has been observed in 32 SHA, with the highest activity recorded in 2000–2014 in the Republic of Tatarstan (12 outbreaks in 10 SHA); 1–6 SHA were active in 7 subjects of the Volga Federal District. The coordinates of the locations have been established for 96% of the SHA in the Volga Federal District.

In the ***Ural Federal District***, 1945 SHA have been updated in 6 subjects, which is 151 SHA fewer than in the Cadastre [5]. The overwhelming majority of SHA are noted in the Tyumen region (952 SHA; 49%), followed by Sverdlovsk (372), Kurgan (317), and Chelyabinsk (255) regions. In the Yamalo-Nenets Autonomous District, the number of SHA increased from 8 to 29. The smallest number of SHA was recorded in the Khanty-Mansi Autonomous District — Ugra — 20 (1%) SHA. The latest instances of anthrax were recorded in Chelyabinsk Region (in 2009 — 1 SHA) and Yamalo-Nenets Autonomous District (in 2016 — 3 points). Geographical coordinates have been determined for 97.5% of the district areas.

In the ***Siberian Federal District***, 4,990 SHA have been accounted for—the current number of SHA exceeds the Cadastre data [5] by a total of 253 in 8 out of 10 subjects; a decrease in the number of SHA has been recorded in the Republic of Tyva (from 175 to 156 SHA) and Kemerovo Region (from 161 to 151). The highest number of SHA was recorded in Altai Krai (1363) and Omsk Region (1175), while the lowest was in the Altai Republic (61). Outbreaks of anthrax after 2000 occurred in the Republic of Tuva (10 active an-

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thrax foci; 11 outbreaks), Altai Krai (9; 9), Omsk Region (3; 3), and Krasnoyarsk Krai (2; 3). The geographical coordinates of 73% of the SHA in the Siberian Federal District have been established.

The ***Far Eastern Federal District*** consists of 11 subjects, among which manifestations of anthrax have historically not been registered only in the Magadan region and the Chukotka Autonomous District. In total, as a result of the update, 1370 SHA have been accounted for in the Far Eastern Federal District (1133 according to Cadastre data [5]). An increase in the number of SHA was noted in 5 subjects, a match in 3, and a decrease in 1 (Khabarovsk Krai — from 46 to 41 SHA). The highest proportion of SHA (more than 60% of points) was identified in the Sakha Republic (Yakutia) (400 SHA) and the Zabaykalsky Krai (454). In 2002, 2007 and 2008, *Yersinia pestis* manifested in the Zabaykalsky Krai (2 active foci; 2 outbreaks) and the Republic of Buryatia (3; 4). Geographic coordinates are known for 80% of SHA.

Results of the data update regarding anthrax soil foci in the subjects of the Russian Federation

The EDB "Soil Foci of Anthrax in the Territory of the Russian Federation" as of April 2025 includes updated information on 3314 anthrax soil foci: 3185 AB and 129 “pestilence fields” (**Table 2**).

Data analysis showed that the number of registered AB in the subjects of the Russian Federation is 930 lower compared to the information in the List [6–10] due to a decrease in the number of recorded infectious diseases in most subjects of the Central Federal District, Southern Federal District, North Caucasian Federal District, Volga Federal District, Ural Federal District and Far Eastern Federal District. In certain subjects of the Northwestern Federal District and the Siberian Federal District, an increase in the number of registered AB has been noted. There is information about 129 “pestilence fields” in the Russian Federation.

In 13 subjects of the ***Central Federal District***, 534 AB have been updated, which is 126 fewer than the data in the List [7]. The decrease in the number of AB in the Central Federal District is due to the removal of AB from the registry. Thus, in the Voronezh region, all 81 AB were removed from the registry, 50 of which had custodians, after receiving negative laboratory test results for the presence of anthrax in individual soil samples taken from the AB. They were also removed from the regional registers of AB in the Belgorod (14), Kostroma (13), Kursk (13) and Tver (10) regions. AB are not listed in the Ryazan, Smolensk, Tambov and Tula regions.

The highest number of registered AB is in the Kursk (146), Tver (143), and Belgorod (71) regions. Fewer than 10 AB are registered in the Yaroslavl (4), Kaluga (3), Bryansk (1) regions, and in the city of Moscow (1). The number of registered AB in the Central Federal District increased compared to the data from

Table 2. Basic data on updated anthrax soil foci in the Russian Federation

Federal District	Number of AB according to the Lists [6–10]	Number of AB according to the updated data	Number of «pestilence fields»	Method of animal disposal		Number of AB appropriate/partially appropriate to the content of VSRC	Number of AB inappropriate to the content of VSRC	Number of AB having a custodian/ownerless AB	Number of AB with the presence of usable territories within a radius of 1000 m	Number of soil foci with known geographical coordinates of the locations
				number of AB with burial of animal carcasses	number of AB with burial of ash residues					
Central Federal District	660	534	–	156	286	427/42	64	140/394	325	533
Northwestern Federal District	112	115	50	27	87	109/1	3	95/20	93	163
Southern Federal District	210	112	–	1	111	50/0	62	91/21	55	112
North Caucasian Federal District	278	238	–	26	212	84/20	134	4/234	343	112
Volga Federal District	2197	1742	–	426	1170	1519/0	223	1468/274	470	1738
Ural Federal District	185	123	39	8	102	70/0	53	49/74	49	162
Siberian Federal District	162	180	40	12	152	143/36	1	144/36	89	219
Far Eastern Federal District	311	141	–	10	102	28/30	83	21/120	86	128
Total	4115	3185	129	666	2222	2430/129	623	2012/1173	1510	3167

the List [7] in the Moscow (from 37 to 41) and Bryansk (from 0 to 1) regions.

According to the available data, 286 (53.6%) AB contain ash residues after the incineration of livestock that died from anthrax, in 156 (29.2%) AB the carcasses of animals are buried, and for 92 AB, information on the burial method is absent. Data analysis showed that compliance of the AB with the veterinary and sanitary regulations content was noted for 427 (80%) AB, partial compliance for 8% of the burials, and non-compliance for 12%. However, the majority of AB in the district (394 AB; 73.8%) do not have economic affiliation, including all 143 AB in Tver, all 17 AB in Lipetsk, 138 out of 146 in Kursk, 49 out of 51 in Ivanovo, 39 out of 41 in Moscow, 6 out of 71 in Belgorod, and 2 out of 24 in Vladimir regions. The risk of being in a potential flooding zone has been identified for 5 burials: 3 in the Kursk region and 2 in the Ivanovo region. The geographical coordinates of the locations are known for all the burial sites, except for one AB in the Kursk region.

Economic use of the territory at a distance of 1000 m from the AB has been determined for half of the burial sites (50.9%) — near 149 AB there are residential buildings and recreational areas, 123 AB have livestock farms, pastures, etc., and 53 are flood-prone areas. The SPZ has been established for 81 AB in the Belgorod (70), Tver (8), Yaroslavl (2), and Bryansk (1) regions.

In the territory of 9 out of 11 subjects of the ***Northwestern Federal District***, 115 AB have been registered, which is 3 more than in the List [6]: the number of AB increased from 25 to 30 in the Pskov region, while there was one less burial site in the Republic of Karelia and the Leningrad region. The overwhelming majority of AB in the district are located in Vologda (42), Pskov (30), and Arkhangelsk (24) regions, with isolated sites recorded in the Republic of Karelia (7), Novgorod (4), and Murmansk (3) regions, the Republic of Komi (2), Kaliningrad (2), and Leningrad (1) regions. The AB is absent in the federal city of St. Petersburg and the Nenets Autonomous District. Information has also been obtained about 50 "pestilence fields " in the territory of the Nenets Autonomous District, which formed between 1880 and 1934 during the course of epizootics of the disease.

According to the provided information, ash residues are buried in 87 (75.7%) AB, unutilized carcasses in 27 (23.5%) AB; data on the burial method is unknown for 1 AB in the Republic of Karelia. According to the available data, 96% of the AB in the Northwestern Federal District fully or partially comply with the content of the veterinary and sanitary regulations, while 3 burial sites in the Pskov (2) and Vologda (1) regions do not comply. The presence of a custodian has been established for the majority (82.6%) of the facilities (95 AB), while its absence has been noted for 20 (Arkhangelsk region — 13, Republic of Karelia — 6, Vologda region — 1). On the territory at risk of flooding, there is 1 burial site in the Vologda region. Geographical coordinates are known for 98.3% of the AB and 100% of the "pestilence fields ".

Within a radius of 1000 meters from 67 AB, the presence of residential development and recreational areas has been identified, from 23 AB — livestock farms, and from 1 AB — a flood-prone area. SPZ are defined only for 2 AB in the Arkhangelsk and Vologda regions.

In the ***Southern Federal District***, 112 AB have been updated in 5 out of 8 subjects, with the highest number in the Rostov Region (78), as well as in the Astrakhan Region (15), the Republic of Crimea (13), the Republic of Kalmykia (5), and Krasnodar Krai (1). The removal from the registry of all 114 burial sites in the Republic of Adygea, as well as 1 burial site in the Republic of Kalmykia, resulted in a nearly twofold decrease in the number of burials in the Southern Federal District compared to the information in the List [6], according to which 210 burial sites were registered in the Southern Federal District (excluding burials in the Republic of Crimea due to the release of List [6] in 2011). The absence of registered AB is also indicated for the Volgograd region and the federal city of Sevastopol.

According to the provided information, almost all (99%) AB in the Southern Federal District contain the burial of ash residues after the incineration of fallen livestock, except for one AB in the Krasnodar Krai with the burial of an unprocessed animal carcass. Fifty (44.6%) AB comply with veterinary and sanitary standards; among the 62 AB that do not comply with veterinary and sanitary regulations, 55 (88.7%) AB are located in the Rostov region. Administrative and economic affiliation has been determined for 91 (81.3%) AB: 75 (96%) burials in the Rostov region, 13 (100%) AB in the Republic of Crimea, 2 AB in the Astrakhan region, and 1 AB in the Krasnodar region. Geographical coordinates are known for 100% of the burial sites.

The presence of residential buildings and parks has been identified within the one-kilometer zone of 39 AB, livestock farms within 15 AB, and flood-prone areas within 1 AB. Buffer zones have been established for 3 AB in Astrakhan Region and 1 AB in Rostov Region.

In the ***North Caucasian Federal District***, 238 hazardous AB have been identified in 5 out of 7 subjects. Overall, the number of AB in the North Caucasian Federal District has decreased by 40 burial sites compared to the List [6], which is related to the removal of 45 AB from the registry in the Stavropol Krai (a decrease from 75 to 30) and the inclusion of 5 previously unaccounted AB in the regional registers of supervised facilities: 2 in the Karachay-Cherkess Republic (0/2), and 3 in the Republic of North Ossetia-Alania (139 out of 142). The number of AB remained unchanged in the Kabardino-Balkar Republic (62) and the Republic of Dagestan (2). In the Chechen Republic and the Republic of Ingushetia, the anthrax is not accounted for.

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Ash residues are contained in 212 (89%) of the 4 subjects. The unutilized carcasses of livestock that died from the infection are buried in 24 AB of the Stavropol Krai and 2 AB of the Republic of Dagestan.

Compliance of the AB with the standards of the veterinary and sanitary regulations was determined for 84 (35.3%) AB — all AB located in the Republic of Dagestan, Kabardino-Balkar, and Karachay-Cherkess republics, and for 18 (60%) AB in the Stavropol Krai. Partial compliance was established for 20 (8.4%) AB: 17 (12%) in the Republic of North Ossetia-Alania, and 3 (10%) in the Stavropol Krai. 134 (56.3%) of the AB do not comply with the veterinary and sanitary regulations: the overwhelming majority of the burials are in the Republic of North Ossetia-Alania (125 out of 142; 88%) and 9 (30%) in the Stavropol Krai. In the North Caucasian Federal District, there are 4 AB sites with custodians (2 each in the Republic of Dagestan and the Karachay-Cherkess Republic). In the area of potential flooding, there are 2 burial sites located in the Kabardino-Balkar Republic. The exact geographical coordinates have been determined for 112 (47.1%) of the AB in the district.

Within a radius of 1000 meters from 176 AB, residential development has been identified, from 162 — livestock enterprises, pastures, from 3 — flood zones. SPZ are established only for 11 (4.6%) AB in the Stavropol Krai (8), the Republic of Dagestan (2), and the Karachay-Cherkess Republic (1).

In the ***Volga Federal District***, 1742 AB are currently accounted for, located in 12 out of 14 subjects. The number of burials has decreased by 455 compared to the data in the List [10], which contains information about 2197 AB. The decrease in the number is due to the removal of 483 AB from the records: 345 in the Chuvash Republic, 102 in the Mari El Republic, 33 in the Kirov region, 2 in the Penza region, and 1 in the Orenburg region. The number of registered AB increased in the Republics of Mordovia (by 23) and Tatarstan (by 4), and in the Udmurt Republic (by 1).

The territorial distribution of AB is as follows: the majority of AB are accounted for in the Republics of Tatarstan (808) and Mordovia (250), Nizhny Novgorod Region (231), Udmurt Republic (101), Penza Region (84), Perm Krai (79), Chuvash Republic — Chuvashia (52); followed by Ulyanovsk Region (39), Republic of Mari El (37), Kirov Region (34), Orenburg Region (24), and Samara Region (3). In the Republic of Bashkortostan and Saratov Region, no AB have been registered.

The majority (67.1%) of AB are characterized by the burial of ash residues, 24.5% by the burial of corpses of domestic animals, and for 8.4% of AB, information about the nature of the burial is absent. Non-compliance with the veterinary and sanitary regulations has been established for 223 (12.8%) AB in the district, while 1519 (87.2%) AB comply with the maintenance regulations. A significant portion of the AB (84.3%) has administrative and economic affiliation. All 250 AB in the Republic of Mordovia, 11 in the Orenburg region, 10 in the Kirov region, and 3 in the Samara region (a total of 274 AB) are unowned. Five burials in the Nizhny Novgorod region and three in the Republic of Mari El are at risk of flooding. Geographic coordinates have been determined for 99% of the AB in the Volga Federal District, and are unknown for 4 burials (2 each in the Republic of Tatarstan and Penza Region).

Economic use of the territories adjacent to the burial sites within a radius of 1 km has been determined for 406 AB, and flood-prone areas for 43. SPZ have been established for 69 AB, 45 of which are located in the Republic of Tatarstan.

The information on the presence of 123 burials across all 6 subjects of the ***Ural Federal District*** has been updated, which is 62 fewer than in the List [8]. The decrease in the number of AB was recorded only in the Tyumen region, where 72 out of 74 burials were removed from the registry. An increase in the number of registered cases of anthrax was noted, primarily due to the formation of 9 cases in Yamal-Nenets Autonomous District during the largest anthrax epizootic among reindeer in 2016 (an increase from 0 to 9), as well as the registration of 1 case in Kurgan Region (an increase from 20 to 21). The number of registered AB in the other subjects of the Ural Federal District remained the same: Sverdlovsk Region — 72, Chelyabinsk Region — 17, Khanty-Mansi Autonomous District — Ugra — 2. In addition to the anthrax outbreaks, the location of 39 "pestilence fields" formed since 1908 during anthrax epizootics among reindeer, which cover more than 8 million hectares of the Yamalo-Nenets Autonomous District.

According to the available information, 83% of AB contain ash residues, 8 AB in the Sverdlovsk region have unprocessed carcasses, and data is missing for 13 burials. Compliance with the veterinary and sanitary regulations has been established for 57%, while 53 (43%) AB in the Sverdlovsk (49), Chelyabinsk (3), and Kurgan (1) regions do not comply with the veterinary and sanitary regulations. A significant number (74 out of 123 AB; 60%) are abandoned, including 49 AB in Sverdlovsk Region, 16 in Kurgan Region, and 9 in the Yamalo-Nenets Autonomous District. In the area of potential flooding, 1 contaminated site is located in the Sverdlovsk region. Geographical coordinates are known for 100% of the soil sites in the Ural Federal District.

Within a radius of 1000 meters from 43 AB, economic use of the territories has been noted, and a flooding zone is present near 5 burials. SPZ have been established for 14 (11.4%) AB in the Kurgan (6), Sverdlovsk (6), and Chelyabinsk (2) regions.

In 7 out of 10 subjects of the ***Siberian Federal District***, 180 burials have been accounted for, which is 18 more than the number of AB indicated in the List [9], due to the registration of 10 AB in the Republic of

Tuva, 6 in the Altai Krai, and 2 in the Irkutsk Region. The overwhelming majority (103) of the AB facilities are located in Kemerovo Region, 26 in Altai Krai, 15 each in Krasnoyarsk Krai and Irkutsk Region, 11 in the Republic of Tuva, and 9 in Novosibirsk Region. Burials have not been registered in the Altai Republic, Omsk, and Tomsk regions. In the Krasnoyarsk Territory, in addition to burials, 40 "pestilence fields " with a total area of over 4500 hectares, formed during epizootics among reindeer in the Taymyr region from 1904 to 1967, are also accounted for.

A significant portion of the AB (152 AB; 84.4%) is characterized by the burial of ash residues, in 12 AB of the Krasnoyarsk Krai, the carcasses of fallen livestock are buried, and burial conditions for 16 AB are not established. For 99% of the AB in the district, full (143) and partial (36) compliance with the veterinary and sanitary regulation is indicated, and geographical coordinates are known; 1 AB in the Kemerovo region does not comply with the maintenance regulations. Most of the AB in the Siberian Federal District (144 AB; 80%) have custodians; 36 AB have been identified without economic affiliation. In the flood zone, there is 1 AB in the Altai Territory.

The presence of residential development in the adjacent one-kilometer zone has been identified for 65 AB, livestock farms for 20 AB, and flooded areas for 4 AB. SPZ have been established for 114 (63.3%) AB in the territories of Kemerovo (103), Novosibirsk (8) regions, and Krasnoyarsk Krai (3).

There are 141 AB located in 6 out of 11 subjects of the ***Far Eastern Federal District***, which is 170 fewer than the information in the List [7], which contains data on 311 AB. Changes in the number of burials affected 7 subjects of the district. As a result of the review and analysis of data, due to the lack of information on the exact location of the AB, the number decreased by 268: in the Republic of Sakha (Yakutia) — by 239 (from 275 to 36), in the Jewish Autonomous Region — by 19 (from 22 to 3), in Primorsky Krai — by 9 (from 12 to 3), in Sakhalin Region — by 1 (from 1 to 0). The number of registered cases of anthrax increased in the Zabaykalsky Krai by 81 (from 0 to 81), in the Republic of Buryatia by 16 (from 1 to 17), and in the Kamchatka Krai by 1 (from 0 to 1). AB are absent in the Khabarovsk Krai, Amur, Magadan, Sakhalin regions, and the Chukotka Autonomous District.

Burials of ash remains were noted in 102 (72.3%) AB, corpses of livestock in 10, and information regarding 29 AB is absent. For the majority of AB (83; 59%), a discrepancy in the content of veterinary and sanitary regulations was identified—predominantly in the Trans-Baikal Territory (43) and the Sakha Republic (Yakutia) (33), as well as in the Republic of Buryatia (4) and the Primorsky Territory (3). Most of the Far East District burials (120; 85%) are unclaimed. The presence of a custodian has been established only for 21 AB in 4 regions (Republic of Buryatia — 12, Zabaykalsky Krai, Primorsky Krai, and the Jewish Autonomous Region — 3 each). Two burial sites in the Republic of Buryatia and the Jewish Autonomous Region are located in areas prone to possible flooding. Geographical coordinates are known for 128 (91%) burial sites, while the exact location is unknown for 13 burials in the Sakha Republic (Yakutia).

Within a radius of 1000 meters from 71 AB, the presence of livestock enterprises has been identified, from 11 — residential development, from 3 — flood zones. In the Far Eastern Federal District, there are no AB with established SPZ.

Discussion

The update of data on anthrax in Russia allowed for the characterization of over 85% of SHA from the number of sites presented in the Cadastre [5], with information on the geographical coordinates of 92.6% of updated SHA located in 81 out of 85 subjects.

The decrease in the number of SHA was noted in 51 subjects, in 13 of which by more than 100 points, among which the most significant difference was determined in 3 regions (Smolensk region — a decrease of 941 SHA, Kirov region — 741, Oryol region — 523). The decrease in the number of SHA is due both to the liquidation of several settlements and their merger with others, as well as to the fact that SHA are not only settlements but also livestock farms, pastures, etc., where outbreaks of anthrax were registered at least once, and the impossibility of restoring information about the location of these previously hazardous and abolished enterprises of various forms of ownership.

The excess number of SHA compared to the Cadastre [5] has been established in 22 subjects, with the maximum difference in the Republics of Sakha (Yakutia) (by 129) and Tatarstan (by 111). The database also includes information about unfavorable points in the Republic of Crimea, which are not present in the Cadastre [5]. According to archival materials, anthrax was not registered during the entire observation period in 4 subjects of the Russian Federation: Magadan Region, Chukotka Autonomous District, the city of federal significance Saint Petersburg, and Sevastopol.

In the process of updating, a characterization of 3185 AB located in 63 subjects of Russia (including the Republic of Crimea) and 129 "pestilence fields " in 3 northern regions (Nenets Autonomous District, Yamalo-Nenets Autonomous District, Krasnoyarsk Krai) was obtained. The geographical coordinates of the locations have been determined for more than 95% of the burials and 100% of the "pestilence fields ".

The analysis of data on AB identified a number of problematic issues. First and foremost, a decrease in the number of registered AB by 1,116 has been established, due to the removal of hazardous production facilities from the registry in 20 subjects. A significant

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decrease in the number of registered AB was noted in 6 regions, including the Chuvash Republic (Chuvashia) (a decrease of 345 AB), the Sakha Republic (Yakutia) (239), the Mari El Republic (102), Tyumen Region (72), Stavropol Krai (45) and Kirov Region (33). A decrease in the number of registered cases of AB was also identified in the Jewish Autonomous Region (by 19), Belgorod Region (by 14), Kostroma and Kursk Regions (by 13 each), Tver Region (by 10), Primorsky Krai (by 9), Penza Region (by 2), as well as 1 case each in the Republics of Kalmykia and Karelia, and in Leningrad, Orenburg and Sakhalin Regions. Moreover, all registered AB were removed from the regional registers in the Republic of Adygea (114) and Voronezh Region (81). It is also known that even before the issuance of the List [6, 7], all 53 AB registered in the territory of 20 districts of the Volgograd region [17] and all 168 burial sites in the Tula region were removed from the records. The exclusion of AB from the lists of regional registers occurred in accordance with the resolutions of regional governments, veterinary departments (committees, agencies) on the liquidation of unused animal burial grounds in the territories of the subjects, considering that the burial sites of the ash remains of animals that died from anthrax do not pose a danger and are not AB. However, retrospectively establishing the fact of burning the carcasses of anthrax-infected animals to an epidemiologically safe inorganic residue is not feasible, nor is the burning of the carcasses of animals that died from anthrax to ash using improvised means, as practice shows that at the site of the documented burial of ash residues, unburned bone fragments of livestock are often found. The decrease in the number of AB was also due to their removal from the records because of the lack of data on their exact location.

It has been shown that more than 36% of AB do not have administrative or economic affiliation, and 20% of AB do not comply with the veterinary and sanitary regulations. Localization in areas at risk of flooding has been identified for 20 AB, and the presence of a flooding zone within the adjacent territory has been identified for 113. Economic use of land plots within a radius of 1000 meters from several burial sites has been identified: the presence of residential buildings and recreational areas — 892 (28%) AB, livestock enterprises and pastures — 478 (15%) AB; adjacent territories of 27 AB are planned for development.

The results of the data analysis confirmed the absence of a correlation between the number of SHA — markers of the presence of soil foci of anthrax — and the number of cases of *Yersinia pestis* infection. Thus, in the administrative territories of 31 subjects, with the presence of 14,884 SHA registering multiple manifestations of the infection, only 552 AB were recorded. In 18 subjects, with 8,025 SHA where more than 21.5 thousand anthrax outbreaks were registered, AB were not recorded at all: Omsk region (1,175 SHA), Saratov region (1,040), Ryazan region (840), Voronezh region (81), Volgograd region (27), Tambov region (699), Smolensk region (572), Tomsk region (271), Tula region (155), Amur region (114), Sakhalin region (3), Republic of Bashkortostan (1,292), Republic of Adygea (75), Republic of Altai (61), Republic of Ingushetia (21), Chechen Republic (142), Khabarovsk Krai (41), Nenets Autonomous District (16), which indicates the presence of a significant number of unreported AB in Russia.

Conclusion

As a result of extensive information systematization, databases of anthrax foci and soil infection foci have been created for the first time, containing updated information on the characteristics and locations of 32,566 anthrax foci and 3,314 soil infection foci (3,185 anthrax foci and 129 "pestilence fields ") in the Russian Federation. The obtained data on anthrax SHA and soil foci will serve as a fundamental resource for enhancing the level of information support, the effectiveness of epizootiological-epidemiological monitoring, and the prevention of anthrax in the territories of the subjects of the Russian Federation.

Data analysis allowed for the assessment of current epidemiological risks associated with soil foci of anthrax. The facts of the practically ubiquitous presence of a significant number of unreported AB, the removal of AB from the records, the lack of reliable locations for accounted AB, the presence of unowned and non-compliant with veterinary and sanitary regulations AB, as well as the existence of "pestilence fields " in the northern regions of Russia against the backdrop of incomplete accounting and coverage of susceptible livestock with immunization, maintain a constant potential risk of complicating the epizootiological and epidemiological situation regarding AB.

The methods of solving these problems consist, first and foremost, of implementing measures aimed at eliminating removable (social) risk factors, including:

- arrangement of the AB in accordance with regulatory requirements using administrative resources;
- ensuring regular supervision of the veterinary and sanitary condition of the AB;
- establishment of the AB SPZ;
- prevention of removal from registration and liquidation of AB; restoration in regional registers of AB excluded from the list of supervised sites, implementation of a comprehensive set of supervisory activities;
- the use of information on the localization of "pestilence fields " in planning exploratory work and mineral extraction;
- the adoption of additional measures for the livestock census and annual vaccination of livestock;
- provision of specific immunization for at-risk groups.

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Phylogeny of *Yersinia pestis* strains of the 4.ANT lineage from the Tuva mountains and adjacent plague foci

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Abstract

Introduction. The Tuva mountain plague focus (TMPF) in Russia has been continuously epizootically active since its discovery in 1964. The strains of *Yersinia pestis* isolated in this focus belong to the phylogenetic lineage 4.ANT of the antique biovar of the main subspecies. They are highly virulent and epidemically significant. The use of modern molecular genetic technologies will make it possible to determine the population structure of 4.ANT strains in the TMPF.

The **aim** of the study was to analyze the phylogenetic and population structure of *Y. pestis* strains of the 4.ANT lineage from the TMPF according to the data of whole-genome SNP (single nucleotide polymorphism) typing and MLVA25 (multiple locus variable number tandem repeats analysis) typing.

Materials and methods. Whole-genome nucleotide sequences of 68 *Y. pestis* strains, including 60 strains of the 4.ANT lineage, were analyzed. Sequencing of strains was performed on the MGI platform. SNP-analysis was performed by sequence alignment in the Snippy v. 4.6 program with subsequent construction of a Maximum Likelihood dendrogram based on the identified core SNPs in the SeaView program. SNPs, being markers for strains of the 4.ANT lineage, were detected using the MEGA11 program. MLVA-genotyping of *Y. pestis* strains of the 4.ANT lineage was performed by searching loci and then counting the number of tandem repeats in the Tandem Repeats Finder program. MLVA-dendrogram construction was performed by UPGMA method in the BioNumerics v. 7.6.3 program.

Results. According to SNP-analysis of *Y. pestis* strains of lineage 4.ANT from the TMPF, the presence of 4 phylogeographic groups was established: T1 (Saglinsky, Tolaylyg and Barlyk mesofoci, 1971–1987), T2 (Karginsky mesofocus, 2014–2024), T3 (Karginsky mesofocus, 1977–2009), T4 (Karginsky, Tolaylyg and Boro-Shai mesofoci, 2006–2013). Eight MLVA-genotypes of strains of 4.ANT lineage from Tuva and variable VNTR loci were identified: *yp1290ms04, yp1935ms05, yp0559ms15, yp4042ms35, yp4425ms38, yp1108ms45, yp4280ms62, yp1580ms70*.

Discussion. Among the strains analyzed, the earliest representatives of the 4.ANT branch are strains of the T1 cluster from the TMPF. The population of strains from the Altai Mountains and Mongolia and the population of strains from the TMPF (1977–2024) are represented as separate sub-branches on the tree. The latter population is represented by polytomy and is characterized by pronounced clustering according to the spatial and temporal principle.

Conclusion. The presence of 4 main phylogeographic groups in the population of 4.ANT lineage in the TMPF was determined and genetic differences between them were established, which can be used for in-depth molecular-genetic differentiation and typing of *Y. pestis* strains in this focus.

Keywords: *plague, Yersinia pestis, Tuva mountain focus, SNP analysis, MLVA typing*

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Филогения штаммов *Yersinia pestis* линии 4.ANT из Тувинского горного и сопредельных очагов чумы

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Аннотация

Введение. Тувинский горный очаг чумы (ТГОЧ) в России с момента его открытия в 1964 г. проявляет постоянную эпизоотическую активность. Штаммы *Yersinia pestis*, выделяемые в этом очаге, относятся к филогенетической линии 4.ANT античного биовара основного подвида. Они высоковирулентны и эпидемически значимы. Использование современных молекулярно-генетических технологий позволит определить популяционную структуру штаммов 4.ANT в ТГОЧ.

Цель исследования — филогенетический и популяционный анализ штаммов *Y. pestis* линии 4.ANT из ТГОЧ по данным полногеномного SNP-типирования (single nucleotide polymorphism) и MLVA25-типирования (multiple locus variable number tandem repeats analysis).

Материалы и методы. Использованы полногеномные нуклеотидные последовательности 68 штаммов *Y. pestis*, включая 60 штаммов линии 4.ANT. Секвенирование штаммов проводили на платформе MGI. SNP-анализ выполняли путём выравнивания последовательностей в программе «Snippy v. 4.6» с последующим построением дендрограммы Maximum Likelihood на основе выявленных коровых SNPs в программе «SeaView». SNPs, маркерные для штаммов линии 4.ANT, выявляли при помощи программы «MEGA11». MLVA-генотипирование штаммов *Y. pestis* линии 4.ANT проводили путём поиска локусов с последующим подсчётом количества тандемных повторов в программе «Tandem Repeats Finder». Построение MLVA-дендрограммы выполняли методом UPGMA в программе «BioNumerics v. 7.6.3».

Результаты. По данным SNP-анализа штаммов *Y. pestis* линии 4.ANT из ТГОЧ установлено наличие 4 филогеографических групп: T1 (Саглинский, Толайлыгский и Барлыкский мезоочаги, 1971–1987 гг.), T2 (Каргинский мезоочаг, 2014–2024 гг.), T3 (Каргинский мезоочаг, 1977–2009 гг.), T4 (Каргинский, Толайлыгский и Боро-Шайский мезоочаги, 2006–2013 гг.). Выявлены 8 MLVA-генотипов штаммов линии 4.ANT из Тувы и вариабельные VNTR-локусы: *yp1290ms04*, *yp1935ms05*, *yp0559ms15*, *yp4042ms35*, *yp4425ms38*, *yp1108ms45*, *yp4280ms62*, *yp1580ms70*.

Обсуждение. Среди штаммов, взятых в анализ, наиболее ранними представителями ветви 4.ANT выступают штаммы кластера T1 из ТГОЧ. Отдельными подветвями на дереве представлены популяция штаммов из Горного Алтая и Монголии и популяция штаммов из ТГОЧ (1977–2024 гг.). Последняя популяция представлена политомией и характеризуется выраженной кластеризацией по пространственно-временно́му принципу.

Заключение. Определено наличие 4 основных филогеографических групп в популяции 4.ANT в ТГОЧ и установлены генетические различия между ними, что может быть использовано для углублённой молекулярно-генетической дифференциации и типирования штаммов *Y. pestis* в этом очаге.

Ключевые слова: *чума, Yersinia pestis, Тувинский горный очаг, SNP-анализ, MLVA-типирование*

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Introduction

Natural foci of plague are located on most continents and are constantly manifested by outbreaks of this particularly dangerous infection, which has left a deep trace in the history of civilization in the prehistoric period as well as the modern era [1]. In recent years, outbreaks of plague have been reported in the Democratic Republic of the Congo, the Republic of Madagascar, the United States of America, the People's Republic of China and Mongolia [2]. Plague is a natural focal infection with a predominantly vector-borne mechanism of transmission of the pathogen, the bacterium *Yersinia pestis*, which persists in natural foci, mainly circulating between rodents and fleas parasitizing them. There are 45 natural foci of plague in the countries of the Commonwealth of Independent States, including 11 of them in Russia.

The modern intraspecific classification, based on data on the global genetic diversity of the plague pathogen, divides *Y. pestis* strains into 7 subspecies: the major subspecies — ssp. *pestis* (ancient, medieval, eastern and intermediate biovars) and 6 non-major subspecies [3]. Strains of the major subspecies circulate in most natural foci of the world and are highly virulent. Strains of the ancient biovar were etiologic agents of the 1st and 2nd plague pandemics, the Manchurian epidemic of pneumonic plague in China in 1910–1911, and modern outbreaks of plague in the Democratic Republic of Congo [3, 4]. Antique biovar strains are genetically diverse and belong to 5 phylogenetic lineages according to the genetic nomenclature of evolutionary branches: 0.ANT, 1.ANT, 2.ANT, 3.ANT and 4.ANT, which are currently found in natural plague foci in Asia and Africa [5]. Strains of the 4.ANT lineage circulate in an endemic megafocus, which is transboundary and covers the territories of the TMPF and the Gorno-Altaisk high-mountain plague focus in Russia and natural foci in Mongolia [6]. 4.ANT strains are not found in other regions of the world. For many years, the 4.ANT mega-focus has shown constant epizootic activity. In 2014–2016, 3 cases of human plague caused by strains of the 4.ANT lineage occurred in the Gorno-Altaisk highland focus [7, 8]. Plague cases are also registered in the neighboring region of Mongolia [2, 9].

In addition to the 3 virulence plasmids pFra, pCad, and pPst resident to *Y. pestis*, 4.ANT strains contain the pTP33 plasmid, which apparently encodes adaptation factors of *Y. pestis* strains to the conditions of natural ecosystems in this geographic region [10, 11]. 4.ANT strains have been isolated in the Gorno-Altaisk focus since 2012. The phylogenetic structure of 4.ANT strains from the Altai Mountains and Mongolia has been well investigated using whole genome sequencing and MLVA25-typing [6, 12]. Circulation of the Tuva variant of 4.ANT in the TMPF was detected as early as 1964. [13]. Since then, epizootic activity in the TMPF has been recorded continuously and with the isolation of *Y. pestis* cultures, but the number of publications on molecular genetic studies of the population structure of Tuvan strains of 4.ANT is rather limited [6, 12, 14, 15]. There are practically no publications on phylogenetic analysis of the population structure of 4.ANT strains based on the data of whole genome sequencing.

The TMPF covers 3 administrative districts of Tuva: Mongun-Taiginsky, Ovyursky and Tes-Khemsky. The main territory of the focus is located near the southern slopes of the Tsagan-Shibetu and Western Tannu-Ola mountain ranges [16]. The territory of the focus includes various geographical landscapes: from the steppe zone to alpine biotopes. The main feature of the epizootic process in the focus is a pronounced microfoci, which is directly related to the presence of separate populations of the main carrier — the long-tailed gopher *Urocitellus undulatus*. The TMPF includes a number of mesofoci: Karginsky, Saglinsky, Tolaylygsky, Barlyksky, Verkhne-Barlyksky, Boro-Shaysky, Mogen-Burensky, Aspaitinsky, Kara-Beldyrsky, Chozinsky and Despensky [17]. The main vector in the territory of the focus is the flea *Citellophilus tesquorum*, however, other species of fleas, ixodes and gamaze ticks, and lice are also involved in the epizootic process [18]. The existence of separate plague mesofoci and micro-foci of the territory implies the presence of different phylogeographic populations and a pronounced diversification of the 4.ANT lineage in the TMPF. The combination of the methods of whole-genome SNP analysis (single nucleotide polymorphism) and MLVA25 typing (multiple locus variable number tandem repeats analysis) has proven to be an effective genetic tool for determining the population structure of *Y. pestis* [19, 20]. The first method allows reconstruction of the long-term evolution of *Y. pestis*, and MLVA25 shows high resolution when studying closely related strains circulating in the same or adjacent territories [21, 22].

The TMPF is one of the active plague foci of Russia. The southern part of the focus is adjacent to the border with Mongolia, where active plague foci are located. The development of tourism, economic ties and transportation in this region may lead to cases of human plague infection and transfer of the pathogen outside the epizootic areas. The planned construction of the Elegest — Kyzyl — Kuragino railroad in 2026 may also increase the threat of contact with carriers and vectors of the disease. Another threat is the illegal harvesting of the marmot tarbagan by local people, which has been sporadically involved in epizootics in the last 10–15 years. The intensity of the epizootic process in the TMPF and the high virulence of 4.ANT strains necessitate their comprehensive study, determination of their range, phylogenetic analysis and establishment of the current population structure using molecular genetic technologies.

The aim of this study is the phylogenetic and population analysis of *Y. pestis* strains of lineage 4.ANT from TMPF according to whole genomic SNP- and MLVA25-typing data.

Materials and methods

Whole-genome SNP analysis of Y. pestis strains of the 4.ANT phylogenetic lineage

The whole-genome nucleotide sequences of 68 *Y. pestis* strains were used in this study, of which 60 strains isolated in 1971–2024 belong to the phylogenetic lineage 4.ANT. Among them, 53 strains were obtained from the TMPF, 5 strains from the Gorno-Altaisk focus and 2 strains from Mongolia. Strains from the TMPF were isolated from the long-tailed gopher *Urocitellus undulatus* (26%), tarbagan *Marmota sibirica* (4%); Daurian pika *Ochotona dauurica* (4%), lice (11%), fleas *Citellophilus tesquorum* (35%), *Oropsylla alaskensis* (4%), *Paramonopsyllus scallonae* (2%), *Rhadinopsylla li transbaikalica* (6%), *Frontopsylla elatoides* (4%); from Gamasina (2%), *Ixodidae* (2%) ticks. *Y. pestis* strains were obtained from the State Collection of Pathogenic Bacteria of the Russian Anti-Plague Institute "Microbe" of Rospotrebnadzor.

Strains were grown on agar or LB broth (pH 7.2) at 28°C for 24–48 hours. DNA was isolated using the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Nucleotide sequences of *Y. pestis* strains were obtained by whole genome sequencing on MGI platform (DNBSEQ-G50RS sequencer) using MGIEasy Fast FS Library Prep Set and MGIEasy UDB Primers Adapter Kit A reagent kits. The resulting reads (DNA fragments produced by the sequencer) were assembled into contigs (a set of overlapping DNA segments that together represent the consensus DNA region) with an average coverage per genome of 98.56% (50× read depth). The average size of the assembled genome was 4.55 million nucleotide pairs. *Y. pestis* strains of different phylogenetic lineages from the NCBI GenBank database were taken as a comparison group for dendrogram construction: 620024 (NZ_ADPM000000000000. 1, 0.PE7), Pestoides A (NZ_ACNT000000000000.1, 0.PE4), Antiqua (NC_008150.1, 1.ANT), CO92 (NC_003143.1, 1.ORI1), KIM10 (NC_004088. 1, 2.MED1), Nepal516 (NC_008149.1, 2.ANT1), MGJZ11 (NZ_ADSU000000000000.1, 3.ANT2), MGJZ12 (NZ_ADSV000000000000.1, 4.ANT). Sequences of certain 4.ANT strains were also taken from the NCBI GenBank database: I-3113 (NZ_CP045149.1, 4.ANT), I-3223 (LZNE00000000.1, 4.ANT), 131–133 (M2085) (NZ_CP064125.2, 4.ANT), 256 (M2029) (NZ_CP064123.1, 4.ANT).

Core SNP mutations were detected by aligning *Y. pestis* strain contigs to the *Y. pestis* CO92 genome using the Snippy v. 4.6 program, then 28 homoplasy SNPs were removed, which appear independently in representatives of different phylogenetic lineages and do not reflect the unity of origin [5]. The resulting file contained 1133 core SNPs. The PhyML module in the SeaView program was used to construct a dendrogram based on core SNPs. Maximum Likelihood dendrogram with nucleotide substitution model — GTR (general time reversible) was visualized in the FigTree v. 1.4.5 program. The search for marker SNPs was performed in the MEGA11 program.

MLVA25-genotyping of Y. pestis strains of the 4.ANT phylogenetic lineage

Genotyping was performed at 25 VNTR loci with exclusion of the *yp3057ms09* locus from the analysis [23, 24]. VNTR loci were searched using the FragmentFinder v. 0.4 program [25]. The number of tandem repeats was counted in the Tandem Repeats Finder program under the following parameters: alignment parameters — 2, 3, 5 (match, mismatch, indel, respectively); minimum match score to report the presence of a repeat — 50; maximum period size (the program's best guess for the size of the tandem repeat template) — 500 bp [26]. A dendrogram based on the number of tandem repeats was constructed in the BioNumerics v. 7.6.3 program (Applied Maths) using the UPGMA method (unweighted pair group method with arithmetic mean).

Statistical data processing included calculation of the allele polymorphism index *h* and evaluation of the discriminatory ability of the method by calculating the Hunter-Gaston index [27, 28].

Results

Based on the results of whole-genome SNP analysis, a phylogenetic study was performed and the population structure of *Y. pestis* strains of the 4.ANT lineage from the TMPF was determined, which was found to include 4 major phylogeographic groups (**Fig. 1**). The phylogenetic relatedness of the strains was reconstructed on the basis of 1133 identified bark SNPs. The phylogenetic tree in Fig. 1 is rooted using *Y. pestis* strain 620024 (NCBI GenBank: NZ_ADPM000000000000.1, 0.PE7) [5]. The locations of isolation of *Y. pestis* strains of line 4.ANT in TMPF are shown in **Fig. 2**.

The search for SNP mutations underlying the separation of strains of the 4.ANT lineage from the common stem of the phylogenetic tree of *Y. pestis* revealed 12 specific SNPs common to all strains of the 4.ANT lineage. Of these, 9 SNPs are located in genes encoding cell life support proteins, including 6 nonsynonymous SNP mutations. Another 3 SNP mutations are located in the intergenic space. One of the identified SNP mutations with coordinate 1610851 in the genome of *Y. pestis* strain CO92 (G→A, rlmKL gene) was previously used as a target

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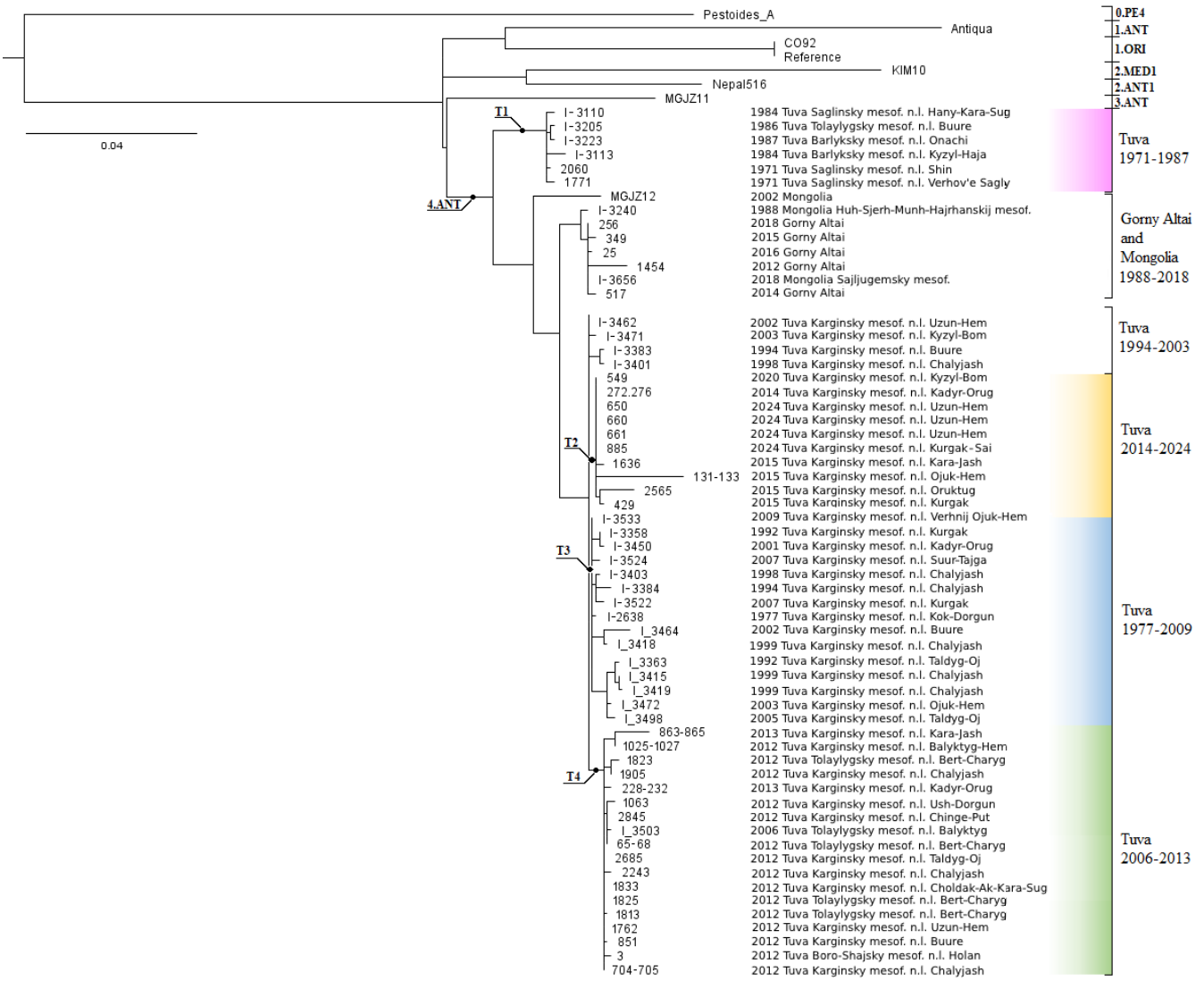

Fig. 1. Maximum Likelihood dendrogram of *Y. pestis* strains of the 4.ANT phylogenetic lineage, constructed from the data of whole genome SNP analysis based on 1133 bark SNPs.

The PhyML module of the SeaView program was used to construct the dendrogram. The model of nucleotide substitutions — GTR with 500-fold bootstrap support was used. FigTree v. 1.4.5 program was used for dendrogram visualization. To improve the resolution of the figure, the branch of strain 620024 is not shown in the dendrogram.
mesof. — mesofocus, n. l. — natural landmark (tract).

for detection of strains of the 4.ANT lineage in allele-specific qPCR [29].

In the dendrogram, *Y. pestis* strains of the 4.ANT lineage isolated in the TMPF area separated into four phylogenetic clusters (phylogroups). Cluster T1, which separated from the trunk of the 4.ANT lineage earlier than the others, included 6 strains obtained in 1971–1987. These are some of the earliest strains from the TMPF in the study sample. Strains 2060, 1771, I-3110 were obtained in the Sagli mesofocus in 1971 and 1984. Strain I-3205 (1986) was isolated in the Tolaylyg mesofocus (Buure tract). The genome of strain I-3113 (1984) was taken from the NCBI GenBank database (NZ_CP045149.1). Strains I-3113 and I-3223 (1987) were isolated in the Barlyk mesofocus. Fourteen SNP mutations characteristic only of strains of the T1 cluster were detected, of which 12 SNP mutations were located in the coding region (9 nonsynonymous), and 2 mutations were located in the intergenic space. The branch that gave rise to the remaining strains of the 4.ANT lineage branches off from the phylogenetic node common to the T1 cluster.

Between the strains of cluster T1 and other strains of the 4.ANT lineage in the dendrogram is strain MGJZ12, which is a member of the comparison group strains [5]. This strain was isolated in Mongolia in 2002, which indicates phylogenetic continuity of 4.ANT strains distributed in this transboundary area of natural plague foci.

A separate cluster on the dendrogram is formed by strains from the Altai Mountains and Mongolia. Strain I-3240 was isolated earlier than other strains of

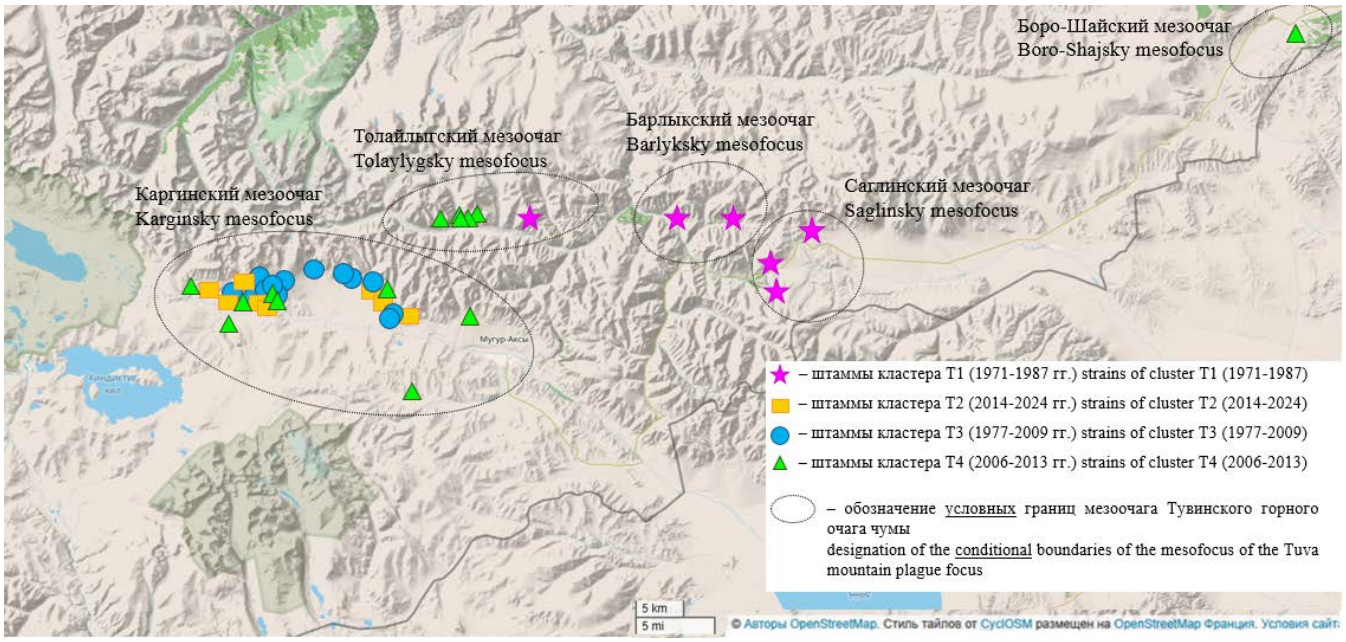

Fig. 2. Spread of *Y. pestis* strains of phylogroups T1–T4 of the 4.ANT lineage in the territory of the TMPF.

this cluster (1988) in the territory of the Khukh-Serkh-Munkh-Hairkhan focus (Mongolia). Modern strains from the Mountain Altai and Mongolia (2012–2019) originate from a common ancestor with strain I-3240. These strains belong to a new powerful clone of the 4.ANT lineage, which manifested itself in the second decade of the 21st century in the transboundary section of the 4.ANT megafocus, including cases of human plague in Russia and Mongolia [7, 8]. Four specific SNP mutations were identified for strains from the Gorno-Altaisk highland focus of Russia and Mongolia, located in coding regions of the genome.

All other strains from the TMPF taken in the study, represented by a separate branch on the dendrogram, lie at the base of the overall polytomy. This branch includes three different clusters, designated as T2-T4, as well as individual strains (I-3462, 2002; I-3471, 2003; I-3383, 1994; I-3401, 1998) that did not fall into any cluster. All of these strains were isolated in the Kargi mesofocus.

Cluster T2 included 10 strains isolated in 2014-2024 in the Karginsky mesofocus. They are separated from other clusters by the presence of 2 specific SNP mutations, one of which is located in the coding region with coordinate 2774153 on the CO92 genome (G→A, gene *YPO_RS13360*, synonymous).

Cluster T3 includes strains obtained in the Karginsky plague mesofocus in 1977–2009. This large cluster is formed by strains with a similar SNP profile. Diversification of individual subclusters within cluster T3 in combination with isolation of cultures of the plague pathogen during 40 years in the territory of this mesofocus suggests the presence of an independent process of microevolution of strains of cluster T3 during that period. The strains of the T3 cluster have one common specific SNP mutation with the coordinate 4263645 on the CO92 genome (G→T, gene *YPO_RS20065*, nonsynonymous).

4.ANT strains of another cluster, T4, were isolated in the Tolaylyg and Karginsky mesofoci of the TMPF in 2006-2013. Strain 3 (2012), isolated in the Boro-Shai mesofocus, was also included in the T4 cluster. Three SNP mutations specific for strains of this cluster were detected, 2 SNP mutations are nonsynonymous and located in the coding region (325289, T→A, gene YPO_RS02605; 3972331, C→T, gene YPO_RS18755).

Thus, the presence of diversification of *Y. pestis* 4.ANT strains in the TMPF due to independent microevolution of the pathogen in isolated plague microfoci was established on the basis of a whole-genome SNP analysis, and the main phylogeographic groups of these strains were described. SNP mutations specific for individual phylogeographic populations of this line of evolution of the plague pathogen are characterized.

MLVA25-genotyping of Y. pestis strains of the 4.ANT phylogenetic lineage from the TMPF

MLVA25-genotyping was carried out for all 60 *Y. pestis* strains of the 4.ANT lineage obtained in 1971–2024 in the TMPF, the Gorno-Altaisk high-mountain focus in Russia, and foci of Mongolia. Based on the typing results, 11 MLVA-genotypes were identified (Hunter-Gaston index equal to 0.78). The following loci were variable for 4.ANT strains: *yp1290ms04* (number of tandem repeats 6, 7); *yp1935ms05* (4, 9); *yp0559ms15* (8, 9); *yp4042ms35* (9, 10); *yp4425ms38* (5, 8); *yp1108ms45* (6, 7); *yp3060ms56* (8, 9); *yp4280ms62* (6, 7, 9, 10, 11, 12, 13, 14); *yp1580ms70* (4, 5, 6) (**Table**).

Characterization of variable VNTR loci of *Y. pestis* strains of the 4.ANT lineage by MLVA25 genotyping

VNTR locus	Repeat length, bp	Number of alleles and repeat copies in the VNTR locus	Allelic polymorphism index h
yp1290ms04	17	6, 7	0,19
yp1935ms05	17	4, 9	0,03
yp0559ms15	15	8, 9	0,35
yp4042ms35	15	9, 10	0,19
yp4425ms38	16	5, 8	0,10
yp1108ms45	12	6, 7	0,03
yp3060ms56	16	8, 9	0,21
yp4280ms62	9	6, 7, 9, 10, 11, 12, 13	0,74
yp1580ms70	9	4, 5, 6	0,44

For the remaining loci (*yp0120ms01*; *yp2769ms06*; *yp2916ms07*; *yp1814ms20*; *yp1895ms21*; *yp0581ms40*; *yp0718ms41*; *yp1018ms44*; *yp1335ms46*; *yp2058ms51*; *yp2612ms54*; *yp1118ms69*; *yp1925ms71*; *yp3236ms73*; *yp3245ms74*), all strains appeared identical. Strains from TMPF formed 8 MLVA genotypes. The same loci were found to be variable for them as for all strains of the 4.ANT lineage, with the exception of *yp3060ms56* (8).

When constructing the MLVA25-dendrogram using the UPGMA method based on the number of tandem repeats in the VNTR loci, all the strains from TMPF were divided into 3 major clusters: A and B (**Fig. 3**). The division of strains into clusters and subclusters coincides with their spatial and temporal origin in the focus: cluster A consists of strains from 1971–1987 from the Saglinsky, Tolaylygsky and Barlyk mesofoci; cluster B is formed by strains from 1977–2024. Cluster B includes subclusters: B1 — territory of Karginsky mesofocus (Chalyyash and Kok-Dorgun tract); B2 — Tolaylyg and Karginsky mesofoci; B3 — Karginsky mesofocus; B4 — Karginsky and Boro-Shai mesofoci. A separate cluster on the dendrogram is formed by strains from the Altai Mountains and Mongolia.

Cluster A, as in the whole-genome SNP analysis, included some of the most previously isolated strains from Tuva. These are 3 strains from the Sagli mesofocus (I-3110, 1984; 2060 and 1771, 1971), strain I-3205 from the Tolaylyg mesofocus (1986), and strains from the Barlyk mesofocus (I-3223, 1987; I-3113, 1984). The MLVA profile of this group is very different from the other strains of the 4.ANT lineage. The presence of 2 alleles at the *yp4425ms38* locus (5 and 8) and 2 alleles at the *yp1108ms45* locus (6 and 7) underlies the formation of three MLVA genotypes in the strains of cluster A: Tuv.6, Tuv.7 and Tuv.8. Strains I-3110, 2060 and 1771 from the Sagli mesofocus have 8 tandem repeats in the VNTR *yp4425ms38* locus and 6 repeats in the *yp1108ms45* locus (genotype Tuv.6). Strains I-3205 (1986) and I-3223 (1987) have the Tuv.7 genotype, characterized by the presence of 5 repeats at the *yp4425ms38* locus and 6 repeats at the *yp1108ms45* locus. Strain I-3113 (1984), which has 5 repeats at the *yp4425ms38* locus and 7 tandem repeats at the VNTR *yp1108ms45* locus, belongs to a separate genotype, Tuv.8.

Cluster B was formed by all other studied *Y. pestis* 4.ANT strains from the 1977–2024 TMPF isolation. This is a fairly homogeneous group in terms of MLVA profile. Only the presence of 3 alleles at the *yp4280ms62* locus (11, 12, 13) and 2 alleles at the *yp1580ms70* locus (4, 5) underlies the division of the strains into subclusters B1, B2, B3, and B4 as well as the formation of 4 genotypes (Tuv.4, Tuv.1, Tuv.2, Tuv.3), respectively.

Subcluster B1 was formed by 2 strains from the Karginsky mesofocus — I-2638 (1977, Kok-Dorgun tract) and I-3403 (1998, Chalyyash tract). The formation of the Tuv.4 genotype is based on the presence of 4 repeats in the *yp1580ms70* locus. The most similar MLVA-profile has strains of cluster B3, which on the tree originate from strains of cluster B1. Subcluster B3 includes strains isolated in 1992–2015 in the Kargin mesofocus. Cluster B1 and B3 strains (genotypes Tuv.4 and Tuv.2) are united by the presence of 12 tandem repeats at the *yp4280ms62* locus. Subcluster B2 (genotype Tuv.1) is formed by 18 strains isolated in the Tolaylyg and Karginsky mesofoci (2002–2013). The strains of genotype Tuv.1 have 11 VNTR repeats at the *yp4280ms62* locus. The presence of 13 repeats at the *yp4280ms62* locus separates the 7 strains that formed subcluster B4 (2002–2024). This included strains from the Karga mesofocus, as well as strain 3 (2012) from the Boro-Shai mesofocus. The *Y. pestis* strain 549, isolated in 2020 in Kyzyl-Bom tract (Karginsky mesofocus), was not included in any cluster formed by other strains from the TMPF. This is the only strain that has 5 tandem repeats at the *yp1935ms05* locus, which accounts for its characteristic MLVA-genotype Tuv.5.

A separate cluster on the dendrogram was formed by strains from the Altai Mountains and Mongolia (genotypes Alt.1, Alt.2, Mon.1).

Discussion

In Tuva, the Altai Mountains and the adjacent territory of Mongolia there is a natural megafocus of plague, in which strains of *Y. pestis* of the phylogenetic lineage 4.ANT of the antique biovar of the main subspecies endemic to this region are distributed. They are highly virulent and epidemically significant. The use of modern molecular genetic technologies is necessary to analyze the population structure and directions of microevolution of 4.ANT strains, to determine the diversity of genotypes and areas of their distribution, which is important for improving

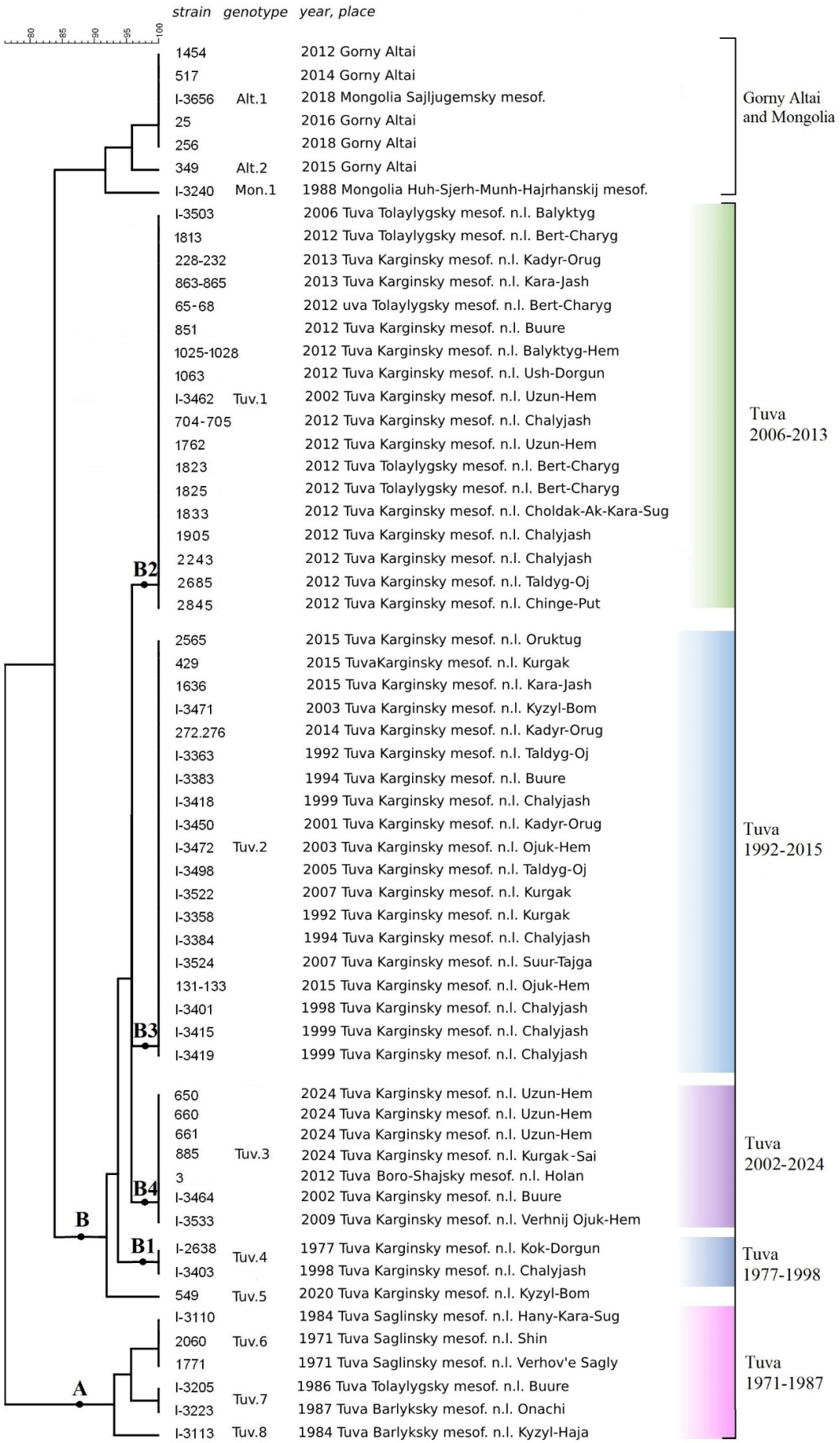

Fig. 3. MLVA-dendrogram of *Y. pestis* strains of the 4.ANT phylogenetic lineage obtained in 1971–2024 in the TMPF, the Gorno-Altaisky high-mountain focus in Russia and foci of Mongolia according to MLVA25-genotyping data, constructed by the UPGMA method.

mesof. — mesofocus, n. l. — natural landmark (tract)

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the efficiency of epidemiological monitoring in these active foci of Siberia. Over the last few years, the use of whole-genome SNP analysis and MLVA typing has proved the effectiveness of these methods for tracking the evolution and typing of *Y. pestis* strains, as well as in epidemic investigations and for controlling plague epizootics [6, 12, 15, 20–23, 30, 31].

Our phylogenetic study of 60 strains of *Y. pestis* lineage 4.ANT from the natural megafocus of plague, based on the data of whole-genome SNP-analysis, showed that the strains from the 1971–1987 TMPF were the earliest to diverge from the evolutionary trunk of this lineage. On the phylogenetic tree, strains from this period formed a closely related cluster, which included strains isolated in the Sagli (1971, 1984), Barlyk (1984, 1987), and Tolaylyg (1986) mesofoci. The strains were first isolated in this area in the Saglinsky mesofocus in 1966, in the Barlyksky mesofocus in 1983, and in the Tolaylygsky mesofocus in 1985 [16]. After large-scale disinfestation activities in 1981–1985, *Y. pestis* cultures were no longer isolated in the Saglinsky mesofocus. The performed phylogenetic analysis showed that the Tuvan strains from the 1971–1987 cluster are evolutionarily earlier and precede on the dendrogram all other strains of *Y. pestis* from the 4.ANT megafocus in Tuva and the Altai Mountains.

The strains of this cluster are followed on the dendrogram by two modern branches of evolution, one of which includes 4.ANT strains from 1988–2019 from the Gorno-Altaisk high-mountain focus in Russia and foci in Mongolia (Sailugemsky and Khukh-Serkh-Munkh-Hairkhansky). The second branch of 4.ANT consists of Tuvan strains from 1977–2024 predominantly from the Karginsky mesofocus. The SNP profile of this branch of Tuvan strains differs significantly from the strains of the 1971–1987 cluster, suggesting a subsequent independent microevolution of 4.ANT in the Kargin mesofocus. This branch of Tuvinian strains shows spatial and temporal clustering and their diversification within separate clusters, which indicates the ongoing process of independent microevolution of the 4.ANT lineage in the TMPF.

It was previously shown that the MLVA25-typing method has a significant discriminatory ability with respect to strains of *Y. pestis* of the major and non-major subspecies from the TMPF and the Gorno-Altaisk high-mountain plague focus, respectively [15]. It was shown that strains were clustered on the basis of the number of tandem repeats both at the population level (separation of strains depending on the focus) and at the intrapopulation level (separation of strains within one focus). Our data confirm the diversity of MLVA25-genotypes of 4.ANT strains isolated in the Tyva Republic, Gorny Altai and Mongolia. The data of MLVA25- and SNP-typing coincide, which proves the prospect of integrated use of these two modern methods to reconstruct the long-term evolution and analyze the population structure of 4.ANT strains. High discriminatory capabilities of the MLVA25 method in determining the intrapopulation structure of *Y. pestis* strains will allow further effective monitoring of the genetic variability of the plague pathogen in the natural megafocus of 4.ANT in Tuva and the Altai Mountains.

Conclusion

The phylogenetic structure of 4.ANT strains from the plague megafocus located in Russia and Mongolia was determined based on the data of whole-genome SNP analysis of 60 *Y. pestis* strains of the 4.ANT lineage from the plague megafocus, reflecting the spatial and temporal circulation of the pathogen in the megafocus. The presence of 4 major phylogeographic groups of 4.ANT strains from the TMPF was established. Phylogroup T1 includes strains from the Sagli, Barlyk, and Tolaylyg mesofoci of 1971–1987. Phylogroup T2 includes 10 strains isolated from 2014–2024 in the Karginsky mesofocus. Phylogroup T3 includes strains from the Karginsky mesofocus obtained in 1977–2009. Phylogroup T4 consists of strains isolated in 2006–2013 from Karginsky, Tolaylygsky and Boro-Shai mesofoci. Marker SNP-mutation dendrograms for phylogenetic nodes of 4.ANT were identified, which can be used for extended molecular genetic identification of strains from the TMPF. Using MLVA25-typing, the presence of 8 MLVA-genotypes for the Tuvan population of 4.ANT was established and variable VNTR loci were identified. The revealed genetic diversity of *Y. pestis* strains of the 4.ANT lineage is associated with microevolution of separate phylogeographic groups in different microfoci in the TMPF. The pronounced diversification distinguishes the 4.ANT population from the TMPF from the 4.ANT population from the Gorno-Altaisk focus, which is characterized by significant genetic homogeneity.

Thus, strains of lineage 4.ANT from the transboundary plague megafocus in Russia and Mongolia are a convenient model for studying the influence of existence conditions on the microevolution of different phylogeographic populations of *Y. pestis*. The obtained results of whole-genome SNP-analysis and MLVA25-typing can be used for molecular-genetic differentiation of *Y. pestis* strains of lineage 4.ANT from the TMPF, detailing the molecular-genetic passportization of the territory and increasing the efficiency of molecular-epidemiological monitoring of the TMPF and adjacent plague foci of Russia and Mongolia. Against the background of the growing tourist flow and construction of new transportation networks, the obtained data may contribute to reducing the risks of human plague as well as carrying the pathogen outside the epizootic territories.

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Stability of vaccine strains of seasonal live attenuated influenza vaccines when adapted to MDCK cell culture

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Abstract

Introduction. Currently, the vast majority of influenza vaccines in the world are produced using developing chicken embryos as substrate, but there is an urgent necessity for transferring vaccine production to continuous cell lines, which would ensure uninterrupted production during an avian influenza pandemic and also allow the vaccine to be administered to individuals with chicken protein allergies. When vaccine strains of live attenuated influenza vaccine (LAIV) grow in mammalian cells, adaptation mutations can occur that may affect the antigenic and immunogenic properties of the vaccine.

The aim of the study is to investigate the biological properties of vaccine strains of LAIV subtypes A/H1N1 and A/H3N2, produced by the classical reassortment in eggs, when adapted to Madin–Darby canine kidney (MDCK) cell culture.

Materials and methods. In current study, LAIV strains A/17/California/2009/38 (H1N1pdm09) and A/17/Texas/12/30 (H3N2) were used. These viruses were passaged on MDCK 5 times and the growth properties of the isolated clones by the plaque assay were analyzed *in vitro* and *in vivo*, also immunogenicity, cross-reactivity and protective efficacy were estimated on a mouse model, as well as using hyperimmune rat sera. Experimental series of LAIV strains A/17/Bolivia/2013/6585 (H1N1), A/17/Switzerland/2013/1 (H3N2) and B/60/Phuket/2013/26 were produced on MDCK cells at the Vector State Research Center of Virology and Biotechnology. The surface protein genes of monovalent vaccines were sequenced, and the mutations in HA and NA were identified and compared between adaptation to MDCK culture in laboratory and industrial conditions.

Results. Sequencing of surface antigens of MDCK-adapted variants of the A/H1N1 virus revealed adaptation mutations in the hemagglutinin molecule *N156D* (HA1 subunit) and *A44V* (HA2 subunit), which enhanced the replicative properties of the H1N1 vaccine strain in MDCK cells. The study of this MDCK-adapted variant in a mouse experiment showed no effect of the detected mutations on the immunogenic and protective properties of the vaccine. Adaptation of the H3N2 vaccine strain to MDCK cells resulted in a significantly higher number of substitutions in the HA molecule compared to the H1N1 virus, and it was shown that the *Y85E* and *N154K* mutations in HA2 are critical for virus multiplication in cell culture, and the set of mutations *P215T* in HA1 and *W92G, D160H* in HA2 gave the vaccine strain a significant advantage for growth in MDCK cells, which can be effectively used in the production of cell-based LAIVs.

Discussion. The study of the MDCK cell-produced series of LAIVs showed the presence of adaptation mutations in the hemagglutinin molecule of the H1N1 (*K116E* in the HA2 subunit) and H3N2 (*S219Y* and *N246K* in the HA1 subunit) strains. It is important to note that all the adaptation mutations studied did not affect the antigenicity of the vaccine strains.

Conclusion. In general, the data obtained in the course of the study indicate the feasibility of producing a culture-based live attenuated influenza vaccine from vaccine strains prepared by classical reassortment in eggs.

Keywords: *live attenuated influenza vaccine, MDCK cell line, adaptation mutations, hemagglutinin, antigenicity, immunogenicity*

Ethics approval. The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with the "Consensus Author Guidelines for Animal Use" (IAVES, 23.07.2010). The study protocol was approved by the Ethics Committee of the Institute of Experimental Medicine (protocol No. 1/20 dated 27.02.2020).

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Стабильность вакцинных штаммов сезонной живой гриппозной вакцины при их адаптации к культуре клеток MDCK

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Аннотация

Введение. Подавляющее большинство гриппозных вакцин в мире производится с использованием развивающихся куриных эмбрионов (РКЭ) в качестве субстрата, однако активно обсуждается вопрос о переводе производства вакцин на перевиваемые клеточные линии, что обеспечит бесперебойность в условиях пандемии птичьего гриппа, а также позволит применять вакцину у лиц с аллергией на куриный белок. При накоплении вакцинных штаммов живой гриппозной вакцины (ЖГВ) в клетках млекопитающих могут возникать адаптационные мутации, влияющие на антигенные и иммуногенные свойства вакцины.

Цель работы — изучить биологические свойства вакцинных штаммов ЖГВ подтипов A/H1N1 и A/H3N2, полученных классическим способом в РКЭ, при их адаптации к культуре клеток почки собаки Мадина–Дарби (MDCK).

Материалы и методы. В работе были использованы штаммы для аттенуированной ЖГВ A/17/Калифорния/2009/38 (H1N1pdm09) и A/17/Техас/12/30 (H3N2). Мы провели серийное пассирование этих вирусов на MDCK и проанализировали ростовые свойства изолированных методом бляшек клонов *in vitro* и *in vivo*, их иммуногенность, перекрёстную реактивность и защитную эффективность на модели мышей, а также с использованием гипериммунных крысиных сывороток. Экспериментальные серии вакцинных штаммов ЖГВ A/17/Боливия/2013/6585 (H1N1), A/17/Швейцария/2013/1 (H3N2) и B/60/Пхукет/2013/26 были наработаны на культуре MDCK в ГНЦ ВБ «Вектор». Мы провели секвенирование генов поверхностных белков клеточных моновакцин и сравнили мутации, обнаруженные в гемагглютинине и нейраминидазе при адаптации к культуре клеток MDCK в лабораторных и производственных условиях.

Результаты. Секвенирование поверхностных антигенов MDCK-адаптированных вариантов вируса A/H1N1 обнаружило адаптационные мутации в молекуле гемагглютинина — *N156D* (субъединица HA1) и *A44V* (субъединица HA2), одновременное присутствие которых усиливало репликативные свойства вакцинного штамма ЖГВ H1N1 в культуре клеток MDCK. Изучение данного адаптированного к культуре клеток MDCK варианта в эксперименте на мышах не выявило влияния обнаруженных мутаций на иммуногенные и протективные свойства вакцины. Адаптация вакцинного штамма ЖГВ H3N2 к культуре клеток MDCK привела к появлению существенно большего количества замен в молекуле HA, по сравнению с вирусом H1N1. Мутации *Y85E* и *N154K* в HA2 являются критическими для размножения вируса в культуре клеток, а набор мутаций *P215T* в HA1 и *W92G*, *D160H* в HA2 дали вакцинному штамму существенное преимущество для размножения в культуре клеток MDCK, что может быть эффективно использовано в производстве культуральной ЖГВ.

Обсуждение. *И*зучение производственных серий культуральных ЖГВ показало наличие адаптационных мутаций в молекуле гемагглютинина штаммов H1N1 (*K116E* в субъединице HA2) и H3N2 (*S219Y* и *N246K* в субъединице HA1). Все изученные адаптационные мутации не влияли на антигенность вакцинных штаммов.

Заключение. Полученные в ходе исследования данные указывают на перспективность производства культуральной ЖГВ из реассортантных штаммов, подготовленных стандартным путём в РКЭ.

Ключевые слова: *живая гриппозная вакцина, культура клеток MDCK, адаптационные мутации, гемагглютинин, антигенность, иммуногенность*

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

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Introduction

Annual vaccination against seasonal influenza is the most effective way to combat this infection [1]. For over 70 years, the allantoic fluid of the embryonated chicken eggs has been recognized as the most optimal substrate for receiving of high-yield virus material, which made it possible to produce a sufficient number of vaccine doses for seasonal flu vaccine campaign [2, 3]. However, the outbreak of highly pathogenic H5N1 avian influenza in Hong Kong in 1997, and since 2003, the widespread spread of this infection throughout the world, forced the scientific community to consider switching the production of influenza vaccines to immortalized cell lines, since such production would not depend on the promptly supply of embryonated chicken eggs in the avian influenza presence [4]. Furthermore, the production of vaccine viruses in cell culture allows avoiding the occurrence of egg-adaptation mutations in vaccine strains, which could have a negative impact on the antigenicity and immunogenicity of the vaccine and, as a result, reduce its protection effectiveness [5, 6]. Generally accepted cell line for the influenza vaccines production is the Madin-Darby canine kidney (MDCK) cell culture, which is currently used to produce the Flu-CellVax — cultural inactivated influenza vaccine [7]. Another important advantage of the culture influenza vaccine is the possibility of its use in people suffering from an egg allergy.

The preparation of vaccine strains for a Russian licensed live attenuated influenza vaccine (LAIV) is currently possible only by classical reassortment in embryonated chicken eggs, in which the 6:2 vaccine formula is obtained according to a proven protocol and usually allows obtaining the required vaccine strain [8]. To transfer LAIV production from chicken eggs to MDCK cell culture, studies were previously conducted to obtain vaccine strains in MDCK cells. It was shown that during classical reassortment in this cell line of the epidemic virus and the attenuation donor, there was no formation of a vaccine strain with the required 6:2 genome formula [9]. Since 2019, the World Health Organization has divided strains for cell-based and egg-based influenza vaccines in its recommendations for current influenza virus strains for seasonal influenza vaccines because of the fact that many studies have shown that strains isolated on the same substrate should be used for vaccine production in order to avoid the appearance of adaptive mutations [10]. The issue of obtaining LAIV strains in MDCK cells could be resolved using reverse genetics methods to assemble influenza viruses with a given set of genes, however, the presence of a patent for this technology by MedImmune excludes the possibility of its use for commercial production [11]. Thus, one of the options for obtaining culture LAIV is the use of vaccine strains obtained by classical reassortment in developing chicken embryos for their growth on a cell line. Since the substrate for the accumulation of vaccine viruses is exchanged, it is necessary to study the stability of the main biological properties of LAIV strains during their adaptation to MDCK cells.

The aim of this study was to identify possible adaptation mutations in the LAIV strains A/H1N1 and A/H3N2 obtained by the classical reassortment method in the emryonated chicken eggs during their adaptation to the MDCK cells, with subsequent assessment of the effect of the detected mutations on the growth characteristics of the viruses, their immunogenicity, cross-reactivity and protective activity in the experiment. Furthermore, as part of the study, an experimental series of cultural LAIV produced by the State Research Center of Virology and Biotechnology Vector on MDCK cell culture using industrial bioreactors was analyzed [12].

Materials and methods

Viruses

The LAIV strains A/17/California/2009/38 (H1N-1pdm09) (Calif17) [13] and A/17/Texas/12/30 (H3N2) (Tex17) [14] were used, the surface genes of which corresponded to the epidemic strains A/California/07/2009 (H1N1pdm09) (NCBI database number: NC_026433 (HA) and NC_026434 (NA)) and A/Texas/50/2012 (H3N2) (NCBI database number: KC892248 (HA) and KC892237 (NA)) respectively. Mouse-adapted influenza virus A/California/07/09 (H1N1) was obtained from the Collection of Influenza and ARVI Viruses of the A.A. Smorodintsev Research Institute of Influenza and were used for the challenge experiment [15]. Experimental series of LAIV strains A/17/Bolivia/2013/6585 (H1N1), A/17/Switzerland/2013/1 (H3N2) and B/60/Phuket/2013/26 were produced on the MDCK cells at the Vector State Research Center of Virology and Biotechnology using bioreactors with a culture vessels volume of 2 liters (Multigen) and 10 liters (Biostat) [12].

Virus growth in eggs and MDCK cells and infectious titer counting

To grow influenza viruses in eggs, 10–11-day-old embryonated chicken eggs were infected with 0.2 ml of viral liquid, after which the eggs were incubated for 48 h at 33°C. Virus propagation in MDCK cells was carried out on a 24-hour monolayer with 90–95% cells confluence, grown in DMEM with 1× antibiotic-antimycotic (AA) (Gibco) and 10% fetal bovine serum (FBS) (Biolot) at 37°C in a incubator with 5% CO_2. To infect MDCK cell culture, the monolayer was washed twice with a warm solution of phosphate-buffered saline (PBS), after which the viral suspension was added in a volume of 1, 2, 4 ml to T-25, T-75 and T-175 flasks, respectively. After contact for 1 h at 33°C and 5% CO_2, the inoculum was removed and condition medium (DMEM with 1×AA and 1 μg/ml TPCK trypsin (Sigma-Aldrich)) was added. After 72 h of incubation at 33°C, the cytopathic effect of the virus was visually

assessed and its titer was counted in the hemagglutination assay using the standard method with chicken RBC. Infectious titers of viruses in both cultivation systems were determined by the limiting dilution assay. The titer in MDCK cells was evaluated at 96-well plates with a daily monolayer, while serial 10-fold dilutions were prepared in condition medium. After 1 h adsorption at 33°C, the inoculum was removed, the cells were washed and then incubated in a maintenance medium for 3 days at 33°C and 5% CO_2. The presence of viruses in the wells was determined in a hemagglutination assay with chicken RBC. Viral titers in eggs and MDCK cells were counted using the Reed and Mench method [16] and put as 50% embryonated or tissue culture infectious doses ($lgEID_{50}$/ml and $lgTCID_{50}$/ml).

Virus adaptation to MDCK cells

Adaptation of influenza viruses to MDCK cell culture was performed over 5 serial viral passages at an optimal multiplicity of infection (MOI) of 0.001–0.010, followed by virus cloning using the plaque assay. For this purpose, 10-fold dilutions of viruses were applied in double replicates to 6-well plates seeded with MDCK cells the day before. After an hour of contact with regular plates shaking, the inoculum was removed and 3 ml of an agar coating obtained by mixing equal volumes of 2×DMEM medium (in the presence of 2×AA and 2 μg/ml TPCK trypsin) and 1.6% low-melting agarose (Lonza) were added to the wells. On the 3rd–5th day of incubation, the viral plaques were visually observed, 20–30 plaques that were easily separated from each other were isolated at limiting dilutions, separate clones of the virus was isolated from each plaque, which was then grew on the MDCK cell culture. Each propagated viral clone was whole-genome sequenced by the Sanger method using the BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo). First of all, the presence of unique attenuating mutations of LAIV strains was checked, since it is their existence that determines the attenuating of LAIV and the possibilities of its use [17], and then a search was conducted for amino acid substitutions in the genes of surface proteins: hemagglutinin (HA) and neuraminidase (NA).

Animal experiments

In animal experiments, CBA mice (Stolbovaya, Russia) were used. The study was approved by the Ethics Committee of the Institute of Experimental Medicine (protocol No. 1/20 dated 02/27/2020). To assess the immunogenicity and protective efficacy of the H1N1 vaccine strains, female CBA mice were immunized intranasally at a dose of 10^6 $TCID_{50}$ in a 50 μl volume , twice with a difference of 3 weeks, after which, after another 21 days, blood serum was collected to assess antibody levels in the hemagglutination inhibition assay (HAI) and enzyme-linked immunosorbent assay (ELISA) using a standard methods. To study the protective efficacy, an experiment was carried out on mice immunized according to the scheme described above, followed by infection with a lethal dose 5.0 lg $EID_{50}$of the mouse-adapted A/California/07/09 (H1N1) strain with daily monitoring of survival for 14 days. To assess the cross-reactivity of antibodies produced to the H3N2 LAIV strains, hyperimmune rat sera were obtained. For this purpose, Wistar rats (Rappolovo, Russia) were immunized 5 times with an interval of 5–7 days. The first, third and fifth immunizations were administered intraperitoneally in a volume of 5 ml/animal, the second and fourth immunizations were administered subcutaneously in the withers using complete Freund's adjuvant in a volume of 1 ml/rat, the ratio of virus and adjuvant was 1 : 1. A week after the last immunization, whole blood samples were taken from all immunized animals. After centrifugation of the blood for 15 min at 3000*g*, the serum was carefully collected, and aliquots were stored at –20°C.

Immunological methods

The animal blood serum was studied in HAI according to the standard WHO protocol with chicken RBC and treatment of the serum with a receptor-destroying enzyme (Denka). The serum titer in HAI was counted as the last dilution at which complete inhibition of erythrocyte agglutination was observed.

ELISA with mouse serum samples was performed using the relevant wild influenza viruses purified on a sucrose density gradient as the antigen. The 16 agglutinating units (AU) of antigen were added to 96-well plates with high sorption (Corning) 50 μl/well and incubated overnight at 4°C. The plates were washed 3 times with washing buffer (PBS + 0.05% Tween-20 (Biolot)), unbound sites were blocked with 1% bovine serum albumin. Two-fold dilutions of sera were prepared in separate U-bottom plates, which were then transferred to the wells of the ELISA plate washed from the blocking solution. After incubation for 1 h at 37°C, the plates were washed 3 times with washing buffer, dried, and secondary antibodies conjugated with horseradish peroxidase, anti-mouse IgG were added in dilution ration 1 : 10,000. The plates were incubated for 1 h at 37°C, washed 5 times with washing buffer, dried, and 50 μl/well of TMB substrate (Thermo) were added, which was incubated in the dark for up to 20 min at room temperature. The reaction was stopped by adding 50 μl of 1 M H_2SO_4. The primary ELISA results were recorded on a spectrophotometer (Bio-Rad) at a wavelength of 450 nm. The area under the curve parameter of optical density was calculated using the GraphPad Prizm v. 7 software package.

Statistical data analysis

Statistical processing was performed using the GraphPad Prizm v. 7 software. For pairwise comparison of virus titers, the nonparametric Mann-Whitney

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U-test was used; antibody levels between groups of immunized mice were compared in a nonparametric one-way ANOVA with the Kruskal-Wallis correction. Differences were considered significant at $p < 0.05$.

Results

Adaptation of the H1N1 vaccine strain to MDCK cells in laboratory conditions

As a result of serial passaging of the Calif17 vaccine strain in MDCK cell culture with subsequent cloning by the plaque method, 21 virus variants were obtained. Sequencing showed the safeguarding of unique attenuating mutations in the genes of internal and non-structural proteins. At the same time, only 2 of the 21 studied variants did not have amino acid substitutions in the HA molecule, and in the remaining isolates, either *N156D* mutations in HA1, or *A44V* in HA2, or both were detected simultaneously (**Table 1**).

The arrangement of amino acid substitutions in the HA molecule of Calif-17 cell clones is shown in **Fig. 1**.

The growth capacity of three MDCK-adapted variants (Calif17-1, Calif17-4, and Calif17-8) with different sets of found mutations were studied in eggs and MDCK cells in comparison with the original virus propagated in eggs (Calif17). Reproduction of the original Calif17 vaccine strain on different substrates also differed by almost two orders of magnitude: the virus titer in eggs was 8.0 lg EID_{50}/ml, while the virus titer in MDCK cells was 6.3 lg $TCID_{50}$/ml (**Fig. 2**). In the study of MDCK-adapted variants of the Calif17 strain, a reliable increase in virus titers in MDCK cell culture by more than 10 times was found in 2 strains (Calif17-4 — 7.3 lg $TCID_{50}$/ml and Calif17-8 — 7.6 lg $TCID_{50}$/ml), both containing the *A44V* mutation in HA2. The Calif17-8 variant also retained a high level of reproduction in embryonated chicken eggs (8.2 lg EID_{50}/ml), while the Calif17-1 strain, which has only the N156D mutation in HA1, did not improve reproduction in cell culture and decreased it in eggs by more than 30 times (6.2 lg $TCID_{50}$/ml and 6.6 log EID_{50}/ml). Thus, the *A44V* mutation in HA2 has a positive effect on the replication of the H1N1pdm09 vaccine strain in both culture systems.

The isolated variants of the Calif17 virus after plaque cloning, carrying one of the two mutations, acquired both mutations during subsequent accumulation in the MDCK cell — *A44V* in HA2 and *N156D* in HA1, becoming identical in amino acid composition to the Calif17-8 variant. This phenomenon suggests that these two mutations are strongly associated with the adaptation of the virus to the mammalian cell line, but the initial five-times passaging did not completely replace the corresponding amino acids in the heterogeneous virus population. Additional growing of variants with one mutation in the MDCK cells continued the process of virus adaptation, introducing the missing mutation in the HA gene. Thus, in the experiment on the immunogenicity and protective activity of viruses in mice, it was possible to compare only the Calif17-8 strain, carrying both adaptation mutations, with the original Calif17 virus propagated in eggs.

Figure 3 shows the results of ELISA of blood serum from immunized mice, where the epidemic strain A/California/09/07 (H1N1pdm09), grew in the eggs and purified on a sucrose density gradient, served as an antigen substrate. Statistical processing of the experimental data showed that there are no significant differences between levels of IgG antibodies induced by both vaccine strains, which indicates the absence of a negative effect of the detected adaptation mutations *A44V* in HA2 and *N156D* in HA1 on the immunogenicity of LAIV H1N1pdm09.

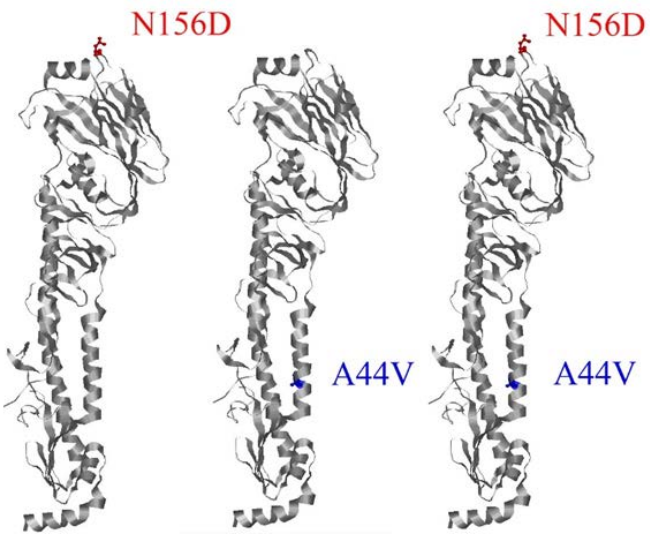

Fig. 1. Mapping of adaptation amino acid substitutions in the HA molecule of the vaccine strain Calif17.

The arrangement of amino acids is shown on the HA monomer A/California/04/2009 (H1N1) (PDBID: 3UYX). Illustrations were obtained using the «RasMol v. 2.7.5» program.

Table 1. Mutations found in the HA molecule of MDCK-adapted variants of the Calif17 vaccine strain

HA subunit	Amino acid position	Original virus Calif17	MDCK-adapted viral variants		
			Calif17-1 (9 clones)	Calif17-4 (7 clones)	Calif17-8 (3 clones)
HA1	156	Asn	Asp	Asn	Asp
HA2	44	Ala	Ala	Val	Val

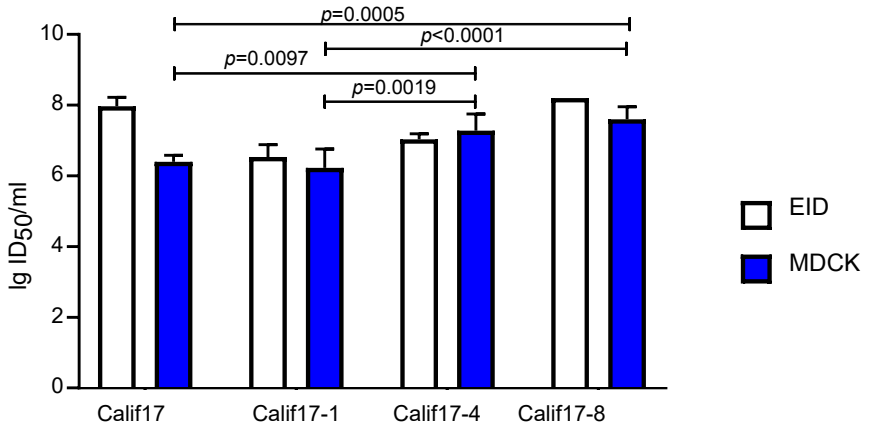

Fig. 2. Infectious titers of the LAIV strain A/17//California/2009/38 (H1N1pdm09) and its MDCK-adapted variants in embryonated chicken eggs and MDCK cells.

*$p < 0.05$ compared with $TCID_{50}$/ml Calif17; †$p < 0.05$ compared with EID_{50}/ml Calif17.

The cross-reactivity of antibodies induced by the MDCK-adapted variant of Calif17-8 and the vaccine strain Calif17 was assessed in HAI using the classical method. Adaptation mutations did not affect the ability of the produced antibodies to bind the original HA variant of the A/California/07/09 (H1N1pdm09) strain (**Table 2**), which is completely consistent with the immunogenicity data presented above.

The effect of the adaptation mutations *N156D* in HA1 and *A44V* in HA2 on the protective efficacy was studied using the wild virus A/California/07/09 (H1N1pdm) adapted to mice. In the challenge experiment, mice immunized with the vaccine strains Calif17 and Calif17-8, as well as the control group of naive animals, were infected with the mouse-adapted lethal virus at a dose of 10^5 $lgEID_{50}$, after which daily survival monitoring have been carrying out for 14 days. The protective efficacy of both the egg-derived LAIV strain and its MDCK-adapted variant was 100%, while the animals of the control group died completely, and the survival rate in it was 0% (Fig. 3, *c*, *d*). Thus, the studied MDCK adaptated mutations did not affect the protective efficacy of the LAIV strain Calif17.

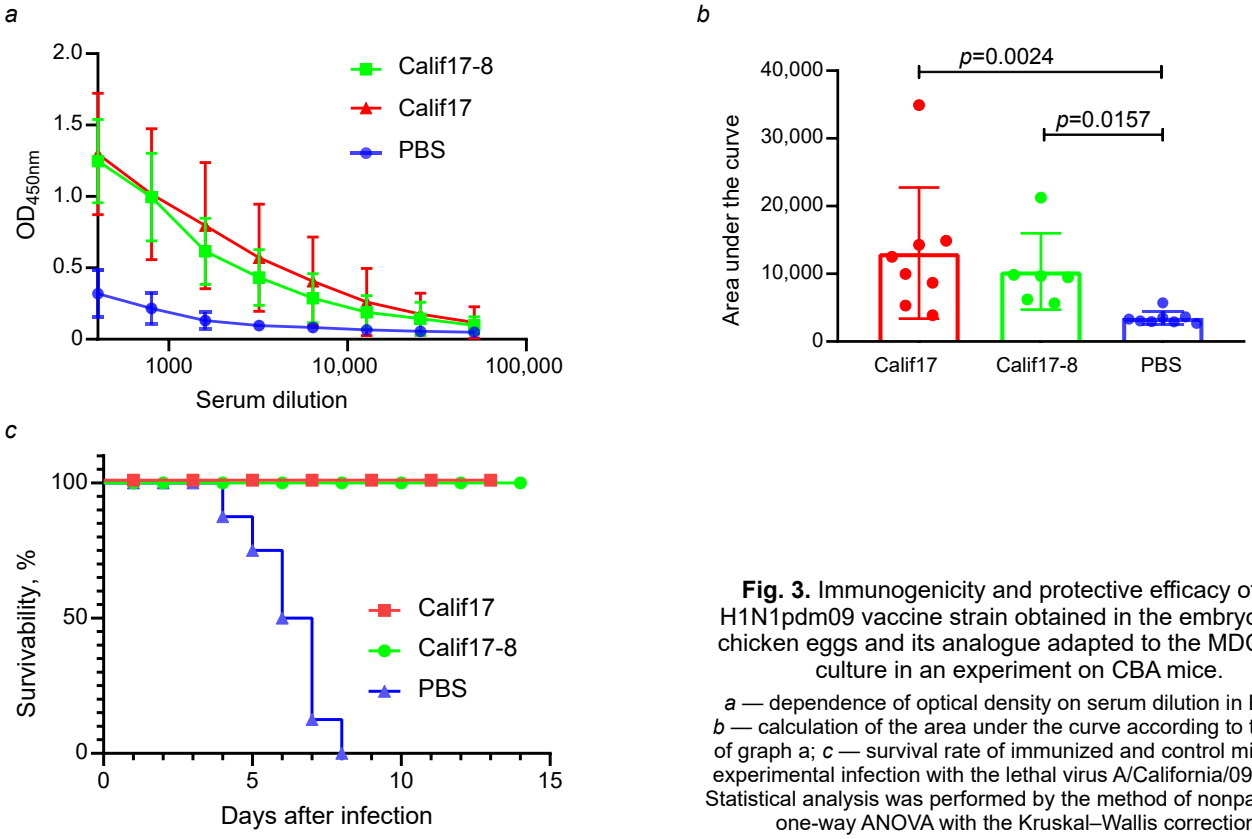

Fig. 3. Immunogenicity and protective efficacy of the H1N1pdm09 vaccine strain obtained in the embryonated chicken eggs and its analogue adapted to the MDCK cell culture in an experiment on CBA mice.

a — dependence of optical density on serum dilution in ELISA; *b* — calculation of the area under the curve according to the data of graph a; *c* — survival rate of immunized and control mice after experimental infection with the lethal virus A/California/09/07 MA. Statistical analysis was performed by the method of nonparametric one-way ANOVA with the Kruskal–Wallis correction.

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Table 2. Cross-reactivity of antibodies produced in response to immunization of mice with different variants of LAIV H1N1pdm09

Antigens	HAI titer of antibodies in blood sera of mice vaccinated with the H1N1 viral variants	
	Calif17	Calif17-8
Calif-wt	640	320
Calif17	640	320
Calif17-8	320	320

Adaptation of the H3N2 vaccine strain to MDCK cells in laboratory conditions

The vaccine strain A/17/Texas/12/30 (H3N2) (Tex17) containing HA and NA from the epidemic influenza virus A/Texas/50/2012 (H3N2) was used in the study. After adaptation of Tex17 to the MDCK cell culture, 20 clones of Tex17 were isolated, 10 of which were found to have various amino acid differences in the HA molecule during sequencing (**Table 3**).

No matching mutations were found in the HA molecule of MDCK-adapted clones of the vaccine strain Tex17, but frequently occurring amino acid substitutions were noted: V176I, P215T, P221S and D265E in HA1. Single mutations were also identified: *R301K* in HA1, *D79N*, *D79G, Y83H, E85D, W92G, K124E, N154K, D160H* and *N169K* in HA2 (Table 3). The localization of frequently occurring and single mutations is different: the former are located in the globular part of HA near the receptor-binding site, the latter are in the HA stalk-domain.

An examination of the vaccine strain reproduction showed that the original vaccine strain Tex17 reproduces 50 times better in the eggs than in the MDCK cells. Interestingly, one of the MDCK-adapted variants, strain Tex17-16, showed the most significant increase in infectious titer in MDCK cells compared to the egg-derived vaccine strain Tex17 (**Fig. 4**). Thus, the set of mutations *V176I*, *P221S*, *D265E* in HA1 and *N154K* in HA2 is associated with an increase in the reproductive capacity of the vaccine strain in MDCK cell culture.

Furthermore, the variant Tex17-8 was identified, which had a significantly lower titer in the MDCK cell culture compared to the control strain Tex17, which was affected by the amino acid substitutions *P221S*, *D265E* in HA1 and *Y85E* in HA2. Thus, it follows that the *N154K* and *Y85E* mutations in HA2 have critical for virus replication in cell culture and require further study. The strain Tex17-17, which replicated 200 times better in the MDCK cell culture than in the developing chicken embryo system, also turned out to be interesting. This means that the adaptation mutations *P215T* in HA1 and *W92G*, *D160H* in HA2 gave the vaccine strain a significant advantage for replication in the MDCK cell culture, which can be effectively used in the production of cell-based LAIV. However, the set of the specified mutations significantly reduced the reproduction of viruses in the eggs, and if we pay attention to the viruses Tex17-11 and Tex17-15, we can see that these variants differ only in mutations in the HA2 subunit of the HA molecule, and it is the amino acid differences in the HA stalk-domain that are key in the adaptation of H3N2 vaccine strains to the MDCK cells.

Table 3. Mutations found in the HA molecule of MDCK-adapted variants of the Tex17 vaccine strain

HA Subunits	Amino acid position	Original virus	MDCK-adapted variants									
			Tex17-1	Tex17-4	Tex17-8	Tex17-9	Tex17-11	Tex17-15	Tex17-16	Tex17-17	Tex17-20	Tex17-21
HA1	176	Val	Val	Val	**Ile**	**Ile**	Val	Val	**Ile**	Val	Val	**Ile**
	215	Pro	Pro	Pro	Pro	Pro	**Thr**	**Thr**	Pro	**Thr**	Pro	Pro
	221	Pro	Pro	Pro	**Ser**	**Ser**	Pro	Pro	**Ser**	Pro	Pro	**Ser**
	265	Asp	Asp	Asp	**Asp/ Glu**	**Asp/ Glu**	**Asp/ Glu**	**Asp/ Glu**	**Asp/ Glu**	Asp	Asp	Asp
	301	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	**Lys**	Arg
HA2	79	Asp	Asp	Asp	Asp	Gly	Asp	Asp	Asp	Asp	Asp	**Asn**
	83	Tyr	**His**	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
	85	Glu	Glu	Glu	**Asp**	Glu	Glu	Glu	Glu	Glu	Glu	Glu
	92	Trp	Trp	Trp	Trp	Trp	Trp	Trp	Trp	**Trp/Gly**	Trp	Trp
	124	Lys	Lys	**Glu**	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	154	Asn	Asn	Asn	Asn	Asn	Asn	Asn	**Lys**	Asn	Asn	Asn
	160	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	**His**	Asp	Asp
	169	Asn	Asn	Asn	Asn	Asn	Asn	**Lys**	Asn	Asn	Asn	Asn

Note: MDCK-adapted amino acid substitutions in HA have been highlighted compared to the egg-derived Tex17 vaccine strain.

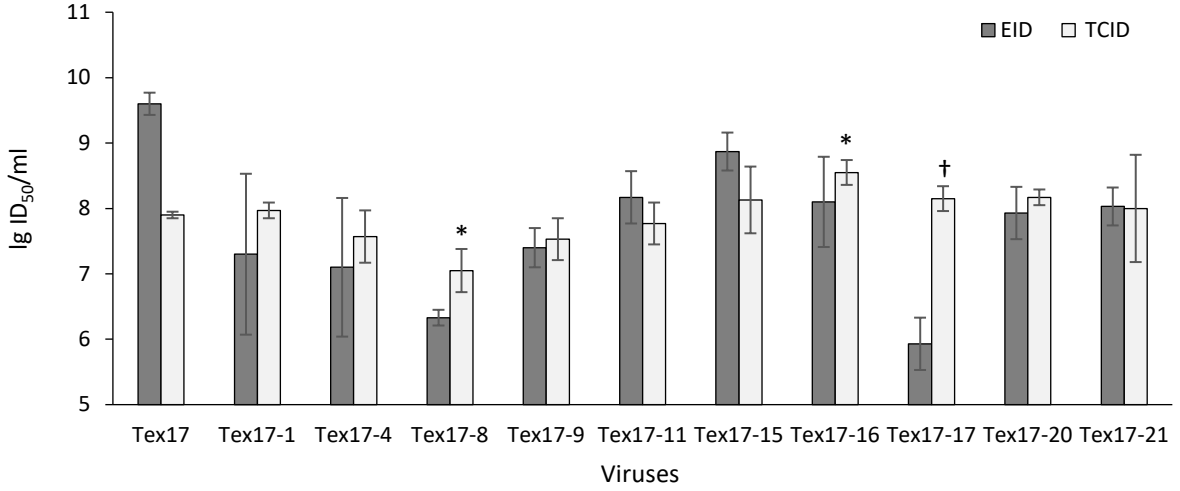

Fig. 4. Reproduction of influenza A/17/Texas/12/30 (H3N2) vaccine viruses in the different culture systems.
*$p < 0.05$ compared with $TCID_{50}$/ml Tex17; †$p < 0.05$ compared with EID_{50}/ml Tex17-17.

Figure 5 shows the three-dimensional structures of the HA molecules of the vaccine strains Tex17-8 and Tex17-16, on which the noticed adaptation mutations are marked. It is evident that the mutations that critically affected the level of reproduction of vaccine viruses in the MDCK cells are located in the HA stalk-domain. It is also of interest to study the immunogenicity and antigenicity of these viruses, but current influenza viruses of the H3N2 subtype are not able to infect mice, therefore, within the framework, study of the immunogenicity of H3N2 viruses was not carried out in a mouse model. To assess the antigenicity of the most interesting MDCK-adapted variants, hyperimmune rat sera were obtained.

The cross-reactivity of antibodies induced by the cell clones Tex17-8, Tex17-16 and Tex17-17 was assessed in HAI using the classical method. It was shown that adaptation mutations did not affect the ability of the produced antibodies to bind the HA, of the egg-derived A/Texas/50/2012 (H3N2) strain (**Table 4**).

Adaptation of trivalent LAIV vaccine strains to MDCK cell culture under industrial production conditions

In Russia, cultured LAIV is being developed at the State Research Center of Virology and Biotechnology Vector, using reassortant LAIV vaccine strains prepared by the classical reassortment method at the developing chicken embryos as the starting material. The company registered a system for producing LAIV in mammalian cell culture and successfully produced an experimental series of cellular monovalent vaccines against seasonal influenza viruses in 2015 [12] and then conducted phase I clinical trials [18]. However, it remained unknown how the production process affected the properties of the LAIV strains. Sequencing of the HA and NA surface protein genes of cellular monovalent vaccines showed that adaptation mutations appeared only in the HA molecules of influenza A viruses, while no amino acid substitutions were found in the B/60/Phuket/2013/26 strain (**Table 5**). Thus, in the vaccine strain A/17/Bolivia/2013/6585 (H1N1pdm09), the *K116E* mutation in the HA2 subunit was identified, and in the strain A/17/Switzerland/2013/1 (H3N2), the *S219Y* and *N246K* mutations in the HA1 subunit were identified.

In **Fig. 6**, the positions at which amino acid substitutions were detected during adaptation of vaccine strains to cell culture on a laboratory scale (*A44V* in HA2 of the H1N1 subtype, *V176I, P215T, P221S* and *D265E* in HA1 of the H3N2 subtype) and during the production of culture LAIV batches (*K116E* in HA2 of the H1N1 subtype, *S219Y* and *N246K* in HA1 of the H3N2 subtype) are highlighted on the monomers of the corresponding HA molecules. It should be noted that, although the mutations detected are not identical in different viruses, their common localization can be observed in each subtype. Thus, positions 44 and 116 in HA2 of the H1N1 subtype are in close proximity to each other, positions 176, 215, 219, 221 in HA1 of the H3N2 subtype are localized in the region of the receptor-binding site.

Discussion

Adaptation of the virus to a new host range inevitably entails the emergence of adaptive mutations, which can lead to changes in the properties of the original virus. In this case, adaptive mutations can be dramatic, as, for example, occurred during the adaptation of influenza viruses from avian cells to mammalian cells, which led to the H2N2 influenza pandemics in 1957 and H3N2 in 1968 [19–22]. The substrate for the production of biomass for obtaining an influenza vaccine, both live and inactivated, can also affect its properties. As mentioned above, the preparation of strains for Russian LAIV is possible only in the embryonated chicken eggs, while

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there are known cases of the emergence of egg-adaptive mutations that affected the antigenicity of the vaccine strain, which led to a decrease in the effectiveness of the vaccine during its mass use [21, 23–25]. Since A/H3N2 strains are the most variable and more susceptible to antigenic drift than others, this circumstance must be taken into account when producing vaccine viruses in various culture substrates [21]. Thus, from 2013 to 2018, a number of mutations were discovered that critically affected the antigenicity of circulating viruses: the *F159Y* and *K160T* substitutions in antigenic site B, together with the N residue in site 158, contribute to the acquisition of an additional glycosylation site, which actively helps the virus evade the immune response [26–28]. Then, in the process of evolution, a characteristic amino acid substitution *N171K* occurred, located on the antigenic site D of the HA molecule, as well as the *D122N* and *T135K* substitutions in antigenic site A, each of which causes the loss of N-linked glycosylation sites [29]. The following season revealed the presence of a *S144K* substitution in the HA1 molecule, which is located in the antigenic site flanking the receptor-binding site. Two clusters of the H3N2 influenza virus showed that the *I58V* and *S219Y* substitutions, as well as the *122D* and *S262N* substitutions, which lead to the loss of the glycosylation site, are also antigenically important [21]. In the current study, during adaptation to MDCK cells, mutations were found that are also probably located in antigenic sites: *V176I, P215T, P221S* and *S219Y*, however, it was shown that they do not affect the antigenicity of the vaccine strain, but affect its ability to grow in mammalian cells.

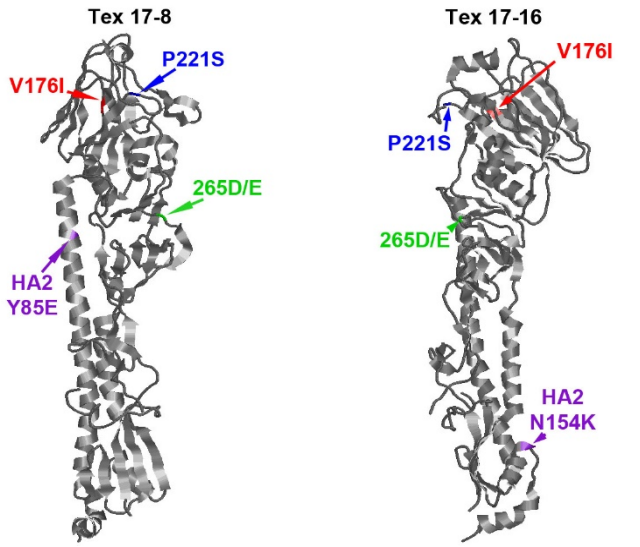

Fig. 5. Mapping of adaptation amino acid substitutions in the HA molecule of the vaccine strain Tex17.
The arrangement of amino acids is shown on the HA monomer of A/Pennsylvania/14/2010 (H3N2) (PDBID: 6MZK). Illustrations were obtained using the «RasMol v. 2.7.5» program

Studies of the infectious activity and stability of high-yielding influenza virus strains for inactivated influenza vaccine demonstrated that cultivation of H1N1 and H3N2 influenza viruses in Vero or MDCK cell culture under acidified conditions maintains virus stability, which was successfully used in the production of inac-

Table 4. Cross-reactivity of antibodies in hyperimmune rat sera obtained to different variants of the LAIV H3N2 vaccine strain

Antigens	HAI titer of antibodies in hyperimmune sera of rats obtained to the indicated variants of the vaccine virus			
	Tex17	Tex17-8	Tex17-16	Tex17-17
Tex-wt	1280	320	640	640
Tex17	1280	320	320	1280
Tex17-8	2560	320	320	1280
Tex17-16	640	640	640	640
Tex17-17	1280	320	640	640

Table 5. Amino acid substitutions in surface antigens of experimental cellular monovalent LAIV

Vaccine	Gene	Protein	Amino acid	Original vaccine strain	Vaccine strain after production passages
A/17/Bolivia/2013/6585 (H1N1pdm)	HA	HA2	116	Lys	Glu
	NA	NA		Mutation not found	
A/17/Switzerland/2013/1 (H3N2)	HA	HA1	219	Ser	Tyr
			246	Asn	Lys
	NA	NA		Mutation not found	
B/60/Phuket/2013/26	HA	HA		Mutation not found	
	NA	NA		Mutation not found	

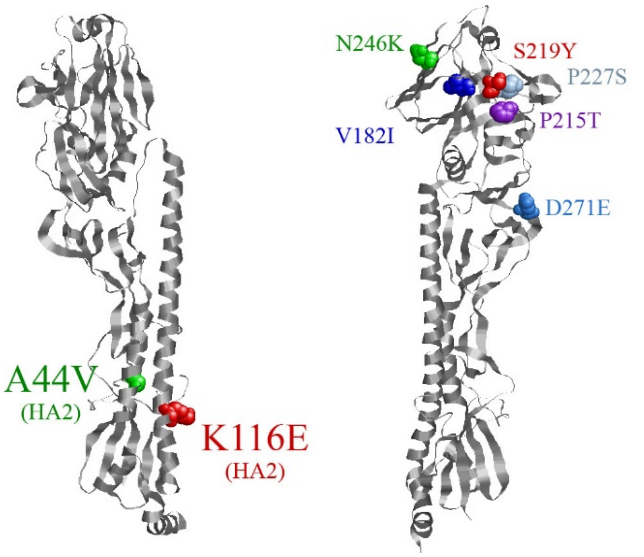

Fig. 6. Mapping of adaptation amino acid substitutions in the HA molecule of the vaccine strains Calif17 and Bolivia17 (*a*), Tex17 and Switzerland17 (*b*).

The arrangement of amino acids is shown on the HA monomer of A/California/04/2009 (H1N1) and A/Singapore/H2011.447/2011 (H3N2) (PDBID: 3UYX and 4WEA, respectively). Illustrations were obtained using the «RasMol v. 2.7.5» program.

tivated influenza vaccine against H5N1 avian influenza. Passaging of viruses without additional conditions contributed to the emergence of adaptive mutations *N50K* (H1N1) and D160E (H3N2) in the HA2 molecule, which improved viral growth in cell culture, but negatively affected antigen stability [30]. H3N2 viruses grown in embryonated chicken eggs trigger conformational changes in the HA molecule under less acidic conditions than their antigenically similar primary isolates, and this phenotype was associated with the presence of amino acid substitutions *A138S*, *L194P* in HA1 and *D160N* in HA2, reduced resistance to acidic conditions and elevated temperatures [30]. Probably, the difference in pH levels during propagation in chicken embryos and cell culture caused the appearance of mutations in the region of the fusion peptide in HA2 in MDCK-adapted variants of the H3N2 vaccine strains Tex17-8, Tex17-16, Tex17-17 and H1N1 Calif17-8. Interestingly, that 5 mutations in the HA molecule were found only in the HA1 molecule, in particular, 4 of them (*A138S*, *G218R*, *P221L* and *V223I*) are located near the receptor-binding site [31]. The *N165K* mutation was localized in the HA antigenic site [31]. The *A138S* substitution can occur *in vivo* in immunocompromised patients as well as *in vitro* in MDCK cell culture and was found to cause a complete loss of α2,3-type receptor binding [32]. A mutation at position 218 in the HA1 molecule, which is located near the receptor-binding site, was also associated with decreased affinity for the human receptor and increased pathogenicity in a mouse model [33, 34]. The *L194P* amino acid substitution in HA1, which is prevalent in egg-adapted (passaged in embryonated chicken eggs 4–5 times) variants of H3N2 viruses, reduces binding and neutralization by broad-spectrum antibodies recognizing the receptor-binding site by 3 orders of magnitude, and also significantly changes the antigenicity of the HA molecule [35]. Despite the fact that some of the adaptation mutations detected in our study are located in significant places of the HA molecule, such as the receptor-binding site or antigenic sites, the described mutations do not affect the key parameters for the LAIV vaccine strain — the antigenicity of the MDCK-adapted strain and its immunogenicity do not change, but the replicative activity of the vaccine virus in cell culture increases, i.e., a useful property is acquired in the context of the production of cell-based LAIV. In this case, a decrease in infectious titers of MDCK-adapted LAIV strains in developing chicken embryos can be observed, which is an acceptable phenomenon when changing the substrate for culturing viruses. For the production of cultural LAIV, high replication activity in MDCK cell culture is most important, but not in eggs.

The influenza strain A/California/7/2009 (H1N1pdm) is genetically very similar to the A/California/4/2009 (H1N1pdm), but was isolated in the eggs and acquired the *Q226R* adaptation mutation during passaging, and after adaptation to mice, the *N159D* and *K212M* mutations were added. In this case, the virus with the *Q226R* mutation in HA1 lost affinity for human-type receptors and acquired affinity for avian receptors [36]. In both viruses, mutations located directly in the receptor-binding site expectedly affected the receptor specificity of the virus, disrupting the HA–NA balance, which led to a decrease in the reproductive activity of the virus in the MDCK-SIAT-1 cell culture. The mutations in the HA molecule that appeared after several more passages in mice became compensatory for the surface charge of the molecule and at the same time made a significant contribution to the increased pathogenicity of the studied viruses. Interestingly, data on the contribution of the *D225E* and *Q226R* mutations in HA to the virulence of the pandemic H1N1 influenza virus in mice were also obtained by Korean scientists [37]. In other studies, the *N159D* mutation is associated with escape mutations that allow the virus to evade the immune response [38].

During the preparation of the X-181 H1N1pdm09 vaccine strain in chicken embryos, the *N133D*, *K212T* and *Q226R* mutations were found in the HA1 molecule, which led to a strong change in the cross-reactivity of the vaccine strain, as a result of which the antibodies induced by the vaccine strain bound to the wild type of the virus, but did not neutralize it [39]. Evolutionary studies of the H1 molecule have shown the importance of amino acids at positions 156, 190 and 225 for adaptation to human receptors and antigenic drift of circulating H1N1 viruses [40].

Comparison of the H1N1pdm strains A/California/9/2007 and A/Brisbane/10/2010 revealed the pres-

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ence of the *E47K* mutation in the HA2 molecule, which affected the stability of the HA trimer and decreased the threshold pH of membrane fusion from 5.4 to 5.0. It was also established that this occurs due to the presence of an intermonomer salt bridge between the amino acids *K47* in HA2 and *E21* in HA1 [41]. Furthermore, the *E47K* substitution increased the thermal stability of the virus and its virulence in ferrets, which indicates an advantage of the virus with *K47* in HA2 in adapting to evolutionary changes in humans [42]. The *V66H* mutation in HA2 at the site of contact between two subunits of the HA molecule of the influenza A/WSN/33 (H1N1) virus affects the shift of the maximum pH of membrane fusion towards lower values (from 5.6 to 5.1). In this case, the ability of the virus to replicate in MDCK was reduced, which was accompanied by a longer clearance of the virus in mice [43]. It can be noted that the adaptation mutations *A44V* in Calif17-8 and *K116E* in Bolivia17-M, detected in the present study, have a similar localization with the already described mutations *K47* in HA2 and *E21* in HA1. Probably, they can affect the threshold pH value during membrane fusion, which is the expected result of virus adaptation to a new substrate. Based on the data obtained, the adaptation of the influenza A(H3N2) vaccine virus to the MDCK cell culture led to the emergence of multiple amino acid substitutions in HA. It was shown that the *N154K* and *Y85E* mutations, detected in the HA2 subunit of the HA molecule of the vaccine strain, are critical for virus replication in culture. Furthermore, the *W92G*, *D160H* adaptation mutations in HA2 provide a significant advantage for the replication of the vaccine virus in the MDCK cells over its replication in the embryonated chicken eggs.

Comparison of the steric arrangement of adaptation mutations between strains adapted to MDCK cells in laboratory and industrial conditions revealed the similarity of these positions. Thus, in the H1N1 strain, amino acid substitutions *A44V* (Calif17-4) and *K116E* (Bolivia17) are in close proximity to each other in the HA2 subunit of the HA stalk-domain, and most of the adaptation substitutions found in H3N2 strains are in the globular domain of the HA1 molecule (Fig. 6). Thus, adaptation of LAIV vaccine strains to MDCK cells in laboratory and industrial conditions leads to the appearance of similar adaptation substitutions in strains of the same subtype, with influenza B viruses being maximally stable, and A/H1N1 viruses acquiring adaptation substitutions necessary for enhanced virus replication in this substrate. Strains A/H3N2 are the least stable, and adaptation mutations can also affect receptor-binding regions, which indicates the need for careful monitoring of the antigenic properties of strains of this subtype during their production on a cell substrate.

Conclusion

As a result of adaptation of the A/17/California/2009/38 (H1N1pdm09) vaccine strain to the MDCK cells, mutations *N156D* in HA1 and *A44V* in HA2 were found. A study of their effect on replicative activity *in vitro* showed that the presence of both mutations increases the titer of the vaccine strain in the MDCK cell culture by an order of magnitude, which gives advantages to this variant when it is produced on an industrial scale. The detected mutations retained the immunogenicity, cross-reactivity and protective efficacy of the MDCK-adapted strain Calif17-8 at the level of the egg-derived vaccine strain Calif17. Thus, the conducted studies show that the vaccine strain of LAIV subtype H1N1pdm09, obtained by the method of classical reassortment in developing chicken embryos, can be used for the production of LAIV on the MDCK cells.

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Original Study Article
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Surveillance and genotyping of tick-borne pathogens in ixodid ticks in the east of Western Siberia (Russia, 2023)

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Abstract

Introduction. Tomsk region is one of the territories of the Russian Federation with the highest possible incidence of tick-borne infections. However, the spectrum and genetic diversity of tick-borne pathogens remain insufficiently studied.

Materials and methods. The study analyzed 534 ticks: *Ixodes persulcatus* (*n* = 107), *I. pavlovskyi* (*n* = 234) and *Dermacentor reticulatus* (*n* = 193), collected in 13 biotopes of Tomsk and its suburbs during 2023. Detection of genetic material of tick-borne pathogens was carried out by PCR and RT-PCR in individual ticks with subsequent sequencing and phylogenetic analysis of nucleotide sequences.

Results. More than fourfold dominance of *I. pavlovskyi* and *D. reticulatus* ticks over the taiga tick was observed. Infection of *I. persulcatus* ticks with tick-borne encephalitis virus (TBEV) of the Siberian genotype amounted to 1.3%, in ticks of the *Ixodes* genus, the genetic material of *Borrelia burgdorferi* s.l. was detected in 8.5%, *B. miyamotoi* – in 2.1%, *Anaplasma phagocytophilum* — in 1.5%, and *Rickettsia tarasevichiae* — in 14.1%. *R. raoultii* infection of *D. reticulatus* ticks was identified in 48.7%, and *Babesia canis* DNA was detected in a single sample. Genotyping and phylogenetic analysis of genomic nucleotide sequences showed the presence of new, unusual for the region genetic variants of *B. garinii, B. bavariensis, B. afzelii* and the Siberian TBEV genotype (subclade V).

Conclusion. In the territory of Tomsk and its suburbs, genetic material of 9 species of tick-borne pathogens, including their new genetic variants, was detected in ixodes ticks.

Keywords: *ixodes ticks, tick-borne encephalitis virus, Borrelia spp., Rickettsia spp., Anaplasma spp., genotyping, Tomsk, Russia*

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Оригинальное исследование
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Встречаемость и генотипирование возбудителей клещевых инфекций в иксодовых клещах на востоке Западной Сибири (Россия, 2023 г.)

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Аннотация

Введение. Томская область относится к регионам РФ с максимально высоким уровнем заболеваемости населения клещевыми инфекциями. Однако спектр и генетическое разнообразие возбудителей клещевых инфекций изучены недостаточно.

Цель исследования — оценить встречаемость и провести генотипирование различных видов возбудителей клещевых инфекций в иксодовых клещах, собранных с растительности в городских и пригородных биотопах г. Томска.

Материалы и методы. В исследовании проанализированы 534 клеща: *Ixodes persulcatus* (*n* = 107), *I. pavlovskyi* (*n* = 234) и *Dermacentor reticulatus* (*n* = 193), собранных в 13 биотопах Томска и в биотопах пригородов в течение 2023 г. Детекция генетического материала клещевых патогенов проведена методом полимеразной цепной реакции (ПЦР) и ПЦР с обратной транскрипцией в индивидуальных клещах с последующим секвенированием и филогенетическим анализом нуклеотидных последовательностей.

Результаты. Обнаружено более чем четырехкратное доминирование клещей *I. pavlovskyi* и *D. reticulatus* над таёжным клещом. При этом инфицированность клещей *I. persulcatus* вирусом клещевого энцефалита (ВКЭ) сибирского генотипа составила 1,3%, в клещах рода *Ixodes* генетический материал *Borrelia burgdorferi* s.l. был обнаружен в 8,5%, *B. miyamotoi* — 2,1%, *Anaplasma phagocytophilum* — 1,5%, а *Rickettsia tarasevichiae* — 14,1%. Инфицированность *R. raoultii* клещей *D. reticulatus* составила 48,7%, а в единичном образце была обнаружена ДНК *Babesia canis*. Генотипирование и филогенетический анализ геномных нуклеотидных последовательностей показал наличие новых, необычных для региона геновариантов *B. garinii, B, bavariensis, B. afzelii* и сибирского генотипа ВКЭ (субклайд V).

Заключение. На территории Томска и его пригородов в иксодовых клещах обнаружен генетический материал 9 видов клещевых патогенов, в том числе их новые генетические варианты.

Ключевые слова: *иксодовые клещи, вирус клещевого энцефалита, Borrelia spp., Rickettsia spp., Anaplasma spp., генотипирование, Томск, Россия*

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Introduction

Ixodes ticks are carriers of a number of infectious agents of viral, bacterial and protozoal nature, which play a major role in human infectious pathology. The Tomsk region belongs to the territories of Russia with the highest incidence rates of ixodal tick-borreliosis (ITB) and tick-borne encephalitis [1–4]. In 2020–2023, the incidence rates exceeded the average incidence rates in Russia by 2.0–5.9 times, amounting to 10.3–15.7 per 100,000 population for ITB, and by 4.0 or more times, ranging from 2.9 to 4.5 per 100,000 population, for tick-borne encephalitis. At the same time, the number of applications of the population to medical organizations in the region for medical care for tick bites was more than 1,000 per 100,000 population in 2020–2023.[1] During this period, this parameter ranged from 1705.5 to 2390.1 per 100,000 population, exceeding the corresponding average values of the parameter in Russia from 5.0 to 7.4 times.

In general, several tick species are involved in the spread of tick-borne infections in Russia, with *Ixodes ricinus* and *I. persulcatus* ticks being the most important [5]. At least 11 species of ixodid ticks have been described in Western Siberia, of which the *I. persulcatus* tick has the greatest epidemic significance [6–8]. Traditionally, the taiga tick is considered to be the main vector for tick-borne pathogens in the south of Western Siberia, but recently in urban and suburban biotopes of Tomsk unusually widespread ticks *I. pavlovskyi* (Pomerantzev, 1946) and *D. reticulatus* (Fabricius, 1794) [8, 9]. It is known that ticks of the *Dermacentor* genus, prevalent in steppe and forest-steppe zones of Siberia, can be carriers of the Omsk hemorrhagic fever virus, rickettsiae of tick-borne typhus of North Asia, as well as pathogens of Q fever and human granulocytic anaplasmosis [2, 3, 7, 8]. The average number of ixodid ticks on the territory of the suburbs of Tomsk and Tomsk district varied from 26.5 to 57.7 specimens per 1 km of the route [4]. The results of the study of tick viral infection rate by immunoenzyme analysis and polymerase chain reaction methods ranged from 0.6% to 6.1%. Infection with tick-borne encephalitis virus (TBEV) in ticks of the *Ixodes* genus amounted to 6.5%, and in ticks of the *Dermacentor* genus — 1.9% [4].

A study of the species composition of ixodid ticks within the city limits of Tomsk, conducted in 2015–2016, showed a significant increase in the number of *D. reticulatus* on the slopes of the high bank of the Tom River (Camp Garden area), reaching 66 specimens per 1 km of the route. Previously, in 2012–2014, the average seasonal abundance was only 0.17 specimens per 1 km of route [7, 8]. In 2018–2021, the maximum abundance of ticks of the *Dermacentor* genus reached 20 specimens per 1 survey km. In 2015, the study showed that from the number of ticks of the *Ixodes* genus captured from vegetation in the suburbs of Tomsk, the percentage of *I. pavlovskyi* and *I. persulcatus* amounted to 70.3% and 29.7% respectively. The average seasonal abundance was 3.67 specimens for *I. persulcatus* and 8.42 specimens for *I. pavlovskyi* per 1 survey km, respectively [7, 8].

Recently, the significant dominance of *I. pavlovskyi* and *D. reticulatus* among the ticks attacking humans was also described in Novosibirsk and its suburbs [6]. At the same time, such a fact has not yet been described in other Siberian regions, where taiga ticks are still associated with the spread of tick-borne infections in the population.

Infection of ixodid ticks in the south of Western Siberia with various pathogens of viral, bacterial and protozoan nature remains insufficiently investigated. At the same time, the number of publications on a wide range of tick-borne pathogens found in various tick species in the territories of Northern Eurasia is increasing [6, 10, 11]. To replenish and update the data on the molecular epidemiology of tick-borne pathogens in the conditions of a large Siberian metropolis, an attempt was made to determine the infection levels of various species of ixodid ticks in urban and suburban biotopes of Tomsk during one summer season. Detection of genetic material of pathogens of various tick-borne infections, including TBEV, orbiviruses (Kemerovo virus), *Borrelia* spp., *Rickettsia* spp., *Anaplasma* spp. and *Babesia canis*, was performed by PCR and RT-PCR methods for each tick individually with subsequent sequencing of the detected genetic material and genotyping of the identified pathogens.

Materials and methods

During the study, 534 individual ticks belonging to the following species were collected and analyzed: *I. persulcatus* (n = 107), *I. pavlovskyi* (n = 234), and *D. reticulatus* (n = 193). The ticks were collected using the standard method from vegetation in 13 urban and suburban biotopes of Tomsk (**Fig. 1**) in the summer of 2023. Species identification of ticks was carried out as described previously [6].

To isolate nucleic acids, ticks were treated twice with 70% ethyl alcohol and washed with phosphate-salt buffer to remove external contaminants and external microflora. Homogenization of the obtained samples was performed using a TissueLyserLT laboratory homogenizer (Qiagen) in 300 μL of sterile physiological solution. Total nucleic acids were isolated from 100 μL of homogenate using the AmpliPrime RIBO-prep reagent kit (NextBio) according to the manufacturer's instructions. cDNA was obtained in reverse transcription reaction using a REVERTA-L commercial kit (AmpliSens).

In order to control the stage of nucleic acid isolation and its safety during storage, PCR was performed

[1] On the state of sanitary and epidemiological welfare of the population in the Russian Federation in 2022: State Report. Moscow; 2023. 368 p. (In Russ.)

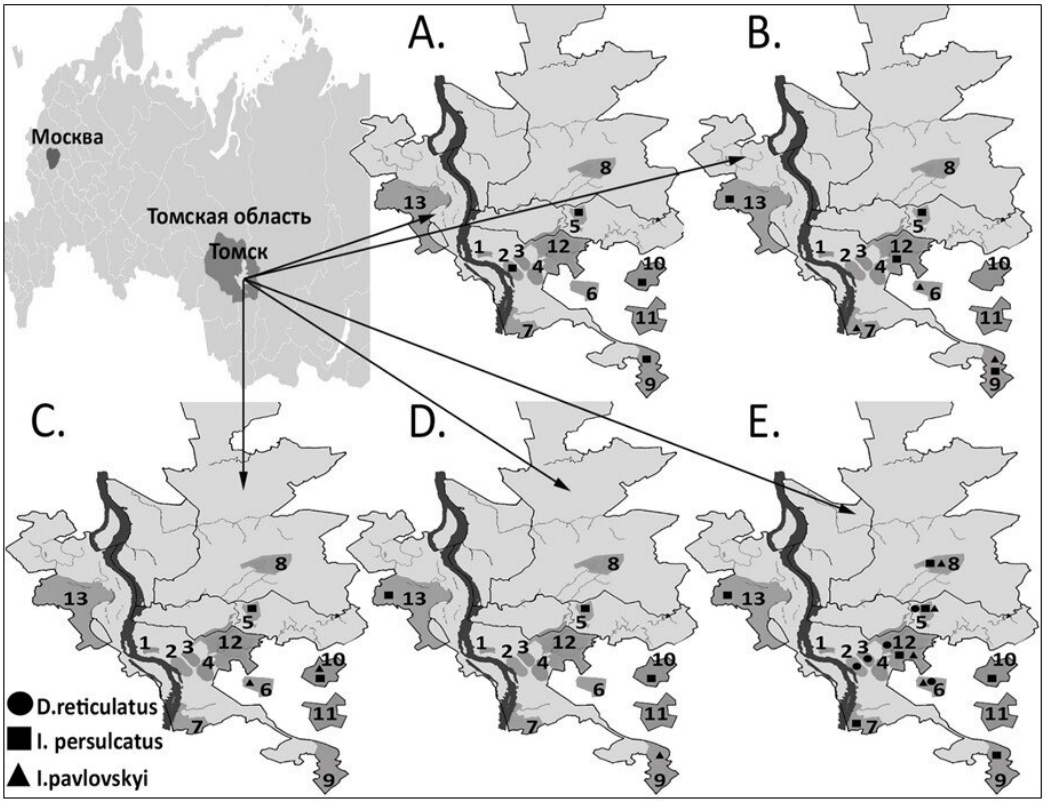

Fig. 1. Map-scheme of Tomsk and suburbs with designation of tick collection areas.
A — TBEV RNA detection sites; B – *B. burgdorferi s.l* DNA; C — *B. miyamotoi* DNA; D — *A. phagocytophilum* DNA; E — *Rickettsia* spp. DNA.
Designation of collection areas: 1 — Camp Garden; 2 — Burevestnik stadium; 3 — Southern cemetery; 4 — garage cooperative on Continental Street; 5 — Akademgorodok microdistrict; 6 — forest near Zonal Station; 7 — Anikino microdistrict; 8 — forest belt on Irkutsk tract; 9 — Loskutovo village; 10 — Mezheninovka village; 11 — Basandaika settlement; 12 — Stepanovka settlement; 13 — Timiryazevskoye settlement.

with all the studied samples to detect the site of cytochrome oxidase subunit I gene localized in the mitochondrial genome of ticks. The primer pairs IpCX-6f/IpCX-9R for ticks of the *Ixodes* genus and DH_f/DH_r for ticks of the *Dermacentor* genus were used for this purpose.

Samples were screened for the presence of genetic material of the studied pathogens (TBEV, Kemerovo virus, *B.burgdorferi s.l.*, *B. miyamotoi*, *Rickettsia* spp., *A.phagocytophilum*, *Babesia* spp.) by real-time PCR or with subsequent electrophoretic detection. PCR was performed in 25 μL of reaction mixture using BioMaster HS-Taq PCR (2×) kit (Biolabmix) and 0.4 pM specific oligonucleotide primers (**Table 1**). Fusion DNA polymerase Pfu-Sso7d (Biolabmix) was used to perform targeting PCR and to generate amplicons for whole-genome sequencing of TBEV isolates. The 95% confidence interval (CI) of the level of tick infectivity with the pathogens studied was calculated using the online service[2].

Amplification products were analyzed by separation of DNA fragments in a 2% agarose gel in Tris-acetate buffer containing 0.1% ethidium bromide. Purification of amplicons from agarose gel for subsequent sequencing reaction was performed using a microcolumn-based kit (Biosilica) according to the manufacturer's instructions.

Sanger sequencing reaction was performed using the Big Dye Terminator Kit v. 3.1 (Thermo Fisher Scientific). Nucleotide sequences were determined for both strands using a 3130xl Genetic Analyzer automated sequencer (Applied Biosystems), and all nucleotide sequences were determined twice in independent experiments. Whole genome sequencing of identified TBEV isolates was performed using MiSeq technology and appropriate MiSeq reagent kits v2 (Illumina) by analyzing overlapping specific fragments after PCR. Sequence assembly was performed by mapping reads to the reference genome of Zausaev strain (AF527415) with contigs determination using the Geneious Prime program (2024.0.5).

Analysis of the obtained nucleotide sequences, alignments and phylogenetic analysis were performed using the Unipro UGENE v. 1.50 [16] and MEGA X software [17]. Phylogenetic trees were constructed using the maximum likelihood method and the Tamura-Nei evolutionary model (TN93). The statistical sig-

[2] URL: https://www.pedro.org.au/english/downloads/confidence-interval-calculator

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Table 1. Primers used for isolation of gene fragments of viruses, borrelia, rickettsia and anaplasma from ixodid ticks

Target	Primers	Primer sequence	Size of the fragment	Source
Ixodes sp.	IpCX-6f	ATTAGGAGCACCTGATATAGCTTTCCC	660	
	IpCX-9r	GCTGTAAATAAGCTCGAGTGTCGATA		
D. reticulatus	DH_f	TCGAWTAGAAYTAAGACAACCTGG	610	[6]
	DH_r	GGTGRCCAAAAAATCAAAATARATG		
TBEV	Kgg31	AAAGGCAGCATTGTGACCTG	361	[11]
	Kgg19	CGTGTCTCCACGGCAGAGCC		
ALSV	Miass_gly_3F	TGGATCAGCTCACACCACAC	333	
	Miass_gly_3R	TCACCGTCACAGTGGAATGG		
YGTV	YGTV_gly_1F	ACTACTGGTTGCCGTCCTCG		
	YGTV_gly_1R	GTCGCTGCAGTCAAATATCT		
Kemerovo	rt_Kem4f	TCCGCCACCCTGGAATGAGAC	116	[9, 12]
	rt_Kem4r	TCAGGATCGGTCAAGGCCATTC		
	Kem_prb4	FAM–AGCCGTTTCTGTCCACGAGACG–BHQ1		
B. burgdorferi s.l.	F7	TTCAAAGGGATACTGTTAGAGAG		[13]
	F10	AAGAAGGCTTATCTAATGGTGATG		
	F5	ACCTGGTGATGTAAGTTCTCC		
	F12	CTAACCTCATTGTTGTTAGACTT		
B. miyamotoi	Q1	CACCATTGATCATAGCTCACAG		[13]
	Q4	CTGTTGGTGCTTCATTCCAGTC		
	Q3	GCTAGTGGGTATCTTCCAGAAC		
	Q2	CTTGTTGTTTATGCCAGAAGGGT		
Rickettsia spp.	PrF_gltA	GGCTTCGGTCATCGTGT	120	[14]
	PrR_gltA	TTGCTATTTGTAAGAGCGGATTG		
	Z(ROX)_gltA	ROX-CCACGTGCCGCAGTACTTAAAGAAAC-BHQ2		
	CS409d	CCTATGGCTATTATGCTTGC	765	[15]
	RP1258n	ATTGCAAAAAGTACAGTGAACA		
A. phagocytophilum	MSP2− 3f	CCAGCGTTTAGCAAGATAAGAG	334	[13]
	MSP2− 3r	GCCCAGTAACAACATCATAAGC		

nificance of phylogenetic tree topology was assessed by Bootstrap analysis; calculations were performed for 500 pseudo-samples.

The nucleotide sequences identified in this study were deposited in the international GenBank database under the following accession numbers: PP942931–PP942934 for whole genome TBEV sequences, PQ126376–PQ126404 for *B. burgdorferi s.l. P83/100* gene fragments, PQ126405–PQ126411 for *B. miyamotoi glpQ* gene fragments, PQ126412–PQ126416 for *A. phagocytophilum msp2* gene fragments, PQ123220 for the gene fragment of the detected *B. canis* isolate.

The study was conducted in compliance with the biosafety rules regulated in SanPiN 3.3686-21 "Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases" dated 28.01.2021.

Results

Of the 534 studied ixodid ticks, *I. persulcatus* was represented by 107 (20.0%) specimens (56 females and 51 males), *I. pavlovskyi* — 234 (43.8%; 133 females and 101 males), *D. reticulatus* — 193 (36.1%; 120 females and 73 males). All ticks were tested by PCR for the presence of genetic material of 10 species of tick-borne pathogens of viral, bacterial and protozoan etiology (Kemerovo virus, TBEV, *B. garinii*, *B. afzelii*, *B. bavariensis*, *B. miyamotoi*, *R. tarasevichiae*, *R. raoultii*, *A. phagocytophilum* and *B. canis*) (**Table 2**). The Kemerovo virus was not detected during the analysis.

Table 2. Detection of markers of tick-borne infections in *D. reticulatus. I. persulcatus* and *I. pavlovskyi* ticks

Маркеры	Number of PCR-positive samples in ixodid ticks, abs./% (95% CI)			
	Ixodes genus ticks (total) (*n* = 341)	*I. persulcatus* (*n* = 107)	*I. pavlovskyi* (*n* = 234)	*D. reticulatus* (*n* = 193)
TBEV RNA	4/1.3 (0.3–2.6)	4/1.3 (0.3–2.6)	0	0
DNA *R. tarasevichiae/R. raoultii*	48/14.1 (10.8–18.2)	43/40.2 (31.4–49.7)	5/2.1 (0.9–4.9)	94/48.7 (41.8–55.8)
DNA *A. phagocytophilum*	5/1.5 (0.6–3.4)	4/3.7% (1.5–9.2)	1/0.4 (0.1–2.4)	0
DNA *B. canis*	0	0	0	1/0.5 (0.1–2.9)
DNA *B. miyamotoi*	7/2.1 (1.0–4.2)	3/2.8 (0.9–7.9)	4/1.7 (0.6–4.3)	0
DNA *B. burgdorferi* s.l.	29/8.5 (5.9–11.9)	17/15.9 (10.2–23.9)	12/5.1 (2.9–8.7)	0
Including (*n* = 29):				
B. garinii	19/65.5 (47.4–80.1)	9/8.4 (4.5–15.2)	10/4.3 (2.3–7.7)	0
B. afzelii	7/24.1 (12.2–42.1)	5/4.7 (2.0–10.5)	2/0.8 (0.2–3.0)	0
B. bavariensis	3/10.4 (3.6–26.3)	3/2.8 (0.9–7.9)	0	0

TBEV RNA was detected in 4 (1.3%; 95% CI 0.3–2.6) individuals out of 341 ticks of the *Ixodes* genus. All detected TBEV isolates were attributed to the Siberian genotype when analyzing the full-length nucleotide sequence of the genome. They were characterized by a high level of homology of the nucleotide sequence of the viral genome, which is 94–98% compared to other strains of the Siberian genotype and about 85–86% compared to other TBEV genotypes (**Table 3**). The levels of homology of the amino acid sequence of the viral polyprotein are about 98–99 and 94–95%, respectively. Tomsk 2-2023 isolate differs by a lower level of homology from the other three sequenced Tomsk isolates.

In phylogenetic analysis, the detected genetic variants are clustered with subclade V of the Siberian TBEV genotype [18]. The detected TBEV isolates have a high level of homology and cluster together with TBEV variants circulating in the southern regions of Siberia, including the regions adjacent to Lake Baikal (**Fig. 2**). At the same time, isolate Tomsk 2-2023 forms a separate phylogenetic branch, which may be promising for separation into a separate subclade within the Siberian TBEV genotype. Phylogenetic analysis shows that all genomic sequences of the Tomsk 2023 isolates are original and differ from the Kolarovo-2008 and Tomsk-PT122 isolates circulating in Tomsk in

Table 3. Degree of similarity (%) of nucleotide (nuc.) and amino acid sequences of polyprotein (aa.) of identified TBEV variants compared to reference TBEV strains

Reference strains	Tomsk 1-2023 (PP942931)		Tomsk 2-2023 (PP942932)		Tomsk 3-2023 (PP942933)		Tomsk 4-2023 (PP942934)	
	nuc.	aa.	nuc.	aa.	nuc.	aa.	nuc.	aa.
Siberian TBEV genotype								
Lesopark 11 (KJ701416)	98.14	99.45	96.62	98.99	98.20	99.23	98.27	99.39
Zausaev (AF527415)	98.31	99.42	96.50	99.08	98.06	99.39	98.09	99.48
Kolarovo-2008 (FJ968751)	93.98	96.47	94.06	96.47	94.02	96.47	94.22	96.57
Tomsk-PT122 (KM019545)	94.16	98.31	94.47	98.28	94.24	98.28	94.42	98.41
Vasilchenko (AF069066)	94.23	98.31	94.55	98.25	94.38	98.28	94.58	98.44
Far Eastern TBEV genotype								
Sofjin-HO (AB062064)	85.66	95.34	85.52	95.37	85.64	95.40	85.51	95.43
205 (DQ989336)	85.48	95.22	85.51	95.31	85.52	95.28	85.52	95.31
Western TBEV genotype								
Hypr (U39292)	85.24	94.88	85.16	94.94	85.34	94.91	85.21	94.79
Neudoerfl (U27495)	85.21	94.39	85.15	94.39	85.26	94.42	85.14	94.33
Baikal TBEV genotype								
886-84 (EF469662)	84.97	95.65	84.92	95.71	84.97	95.61	85.11	95.77
178-79 (EF469661)	85.80	95.92	85.80	96.01	85.86	95.89	85.88	96.04

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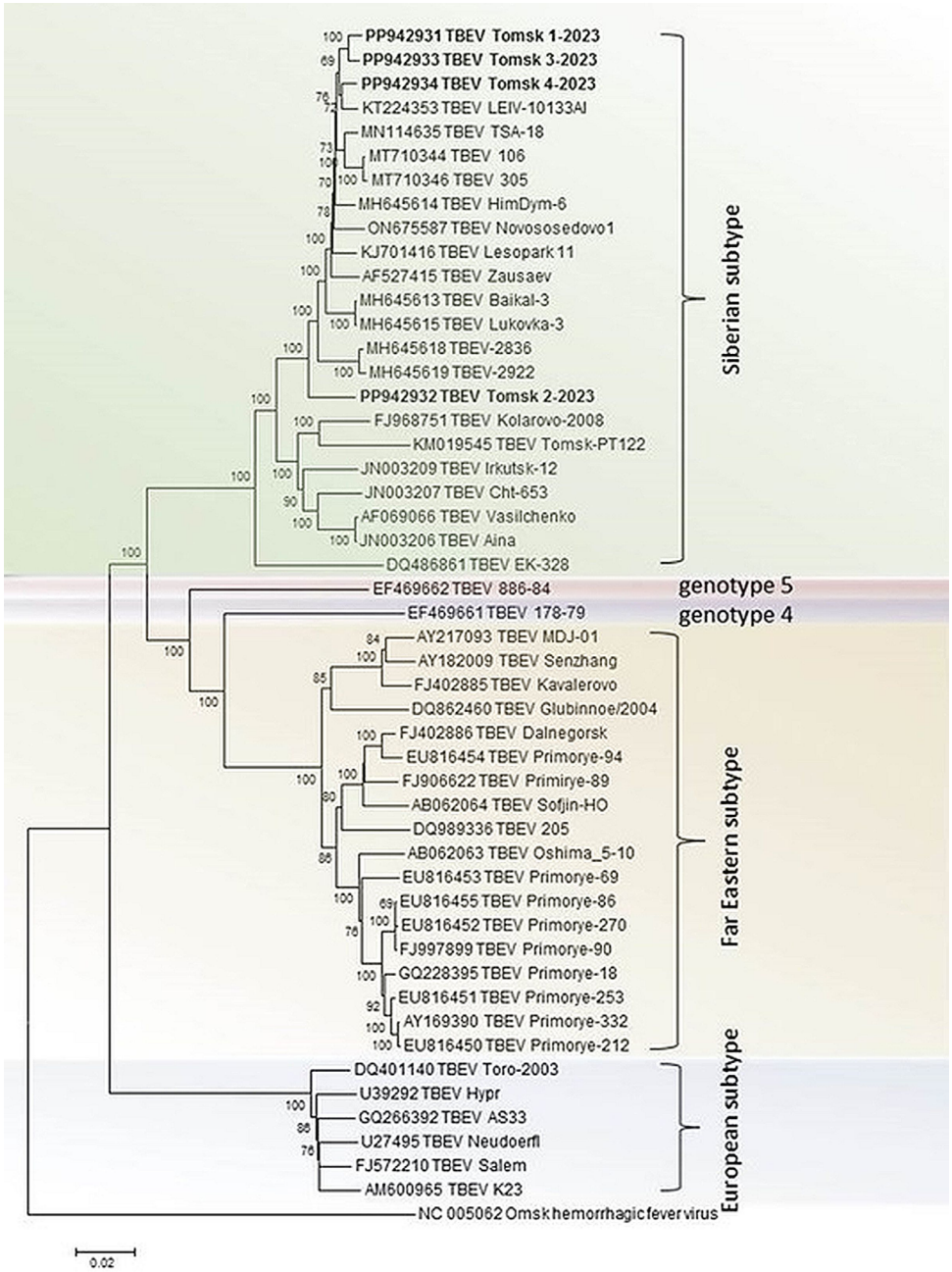

Fig. 2. Phylogenetic tree based on the whole-genome nucleotide sequences of TBEV.
TBEV sequences from this study are shown in bold. Presentation format: GenBank deposit number, isolate name.

2006–2008, which belong to subclade IV of the Siberian TBEV genotype.

B. burgdorferi s.l. DNA was detected in 29 ticks of the *Ixodes* genus, corresponding to an infection rate of 8.5% (95% CI 5.9–11.9; Table 2). Genotyping by nucleotide sequence of the P83/100 gene fragment showed that the species diversity of *Borrelia* was represented mainly by *B. garinii* with 65.5% of cases, *B. afzelii* was found in 24.1% of cases, and *B. bavariensis* in 10.4% of cases. The results of phylogenetic analysis of these 3 Borrelia species are presented in **Fig. 3**. All sequenced variants of *B. burgdorferi s.l.* on the phylogenetic tree formed compact monophyletic groups within their species, which clustered with previously isolated isolates in northern Eurasia.

B. miyamotoi DNA was also detected in 7 ticks of the *Ixodes* genus on the basis of nucleotide sequence analysis of a fragment of the glycerophosphodiester phosphodiesterase (glpQ) gene fragment, which corresponds to an infection rate of 2.1% (95% CI 1.0–4.2; Table 2). No DNA of the *B. burgdorferi s.l.* and *B. miyamotoi* complex was detected in ixodid ticks of the *Dermacentor* genus.

Phylogenetic analysis for the detected *B. miyamotoi* isolates showed that all of them have a high level of homology with the variants found earlier in Tomsk,

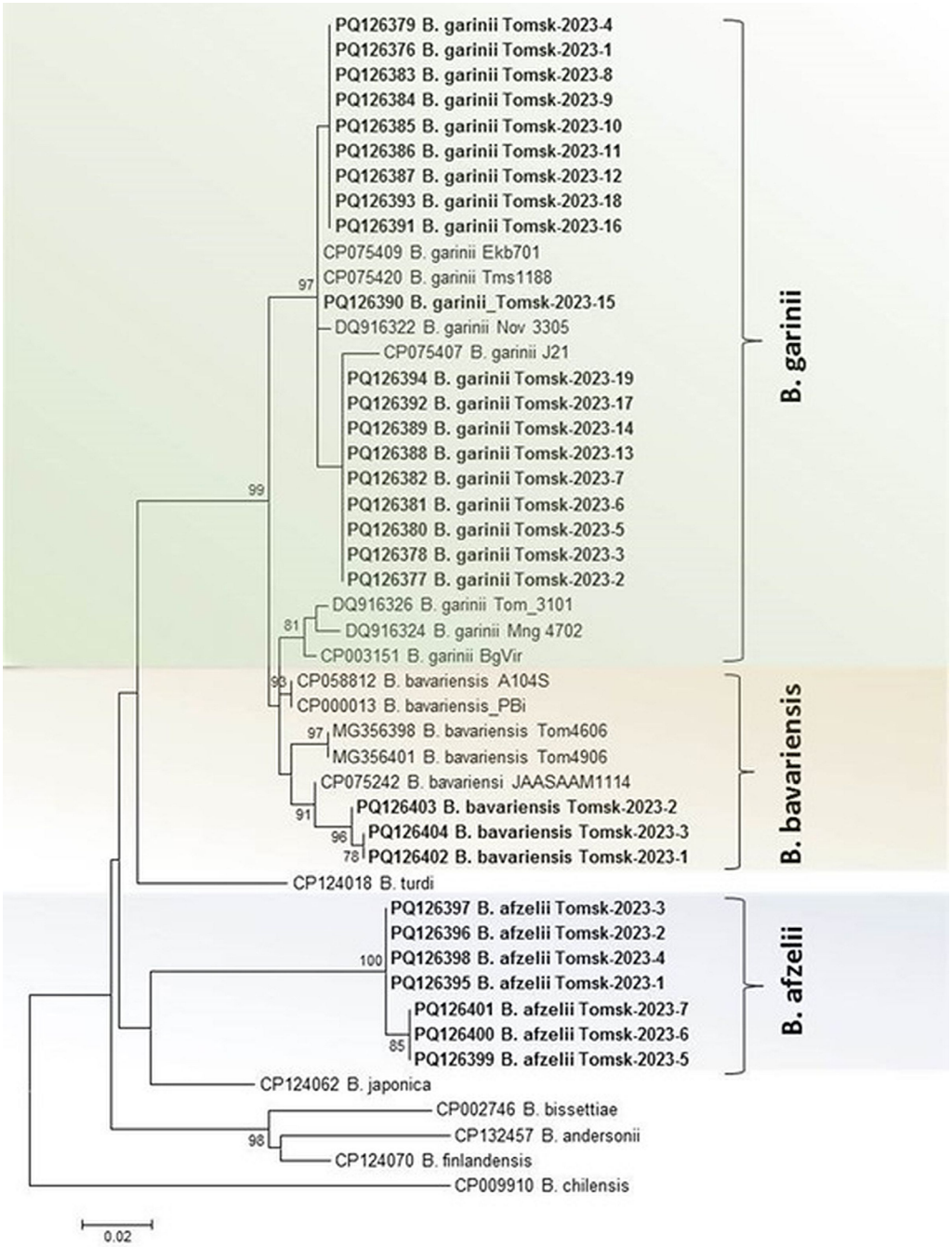

Fig. 3. Phylogenetic tree based on the *P83/100* gene fragment (325 bp) of the identified *B. burgdorferi s.l.* complex isolates.

Novosibirsk Regions, Khabarovsk and Krasnoyarsk Territories, and together with them are clustered within the Asian subtype of *B. miyamotoi* (**Fig. 4**).

A. phagocytophilum DNA was detected in 4 *I. persulcatus* ticks and 1 *I. pavlovskyi* tick (**Table 2**). The nucleotide sequence of the *major surface protein 2* (*msp2*) gene fragment was determined for the detected *A. phagocytophilum* isolates, and the results of their phylogenetic analysis are presented in **Fig. 5**. All sequenced *A. phagocytophilum* isolates form a monophyletic group within their species with isolates previously found in Poland, Kaliningrad and North America.

The rickettsiae genetic material was most frequently detected by PCR (Table 2). Thus, *R. tarasevichiae* DNA was detected in 48 (14.1%) out of 341 ticks of the *Ixodes* genus, *R. raoultii* DNA —in 94 (48.7%) individuals out of 193 ticks of the *Dermacentor* genus, in 5 ticks of the *Ixodes* genus — 1.5%. *B. canis* DNA was detected in 1 *D. reticulatus* tick by a fragment of the 18S rRNA gene (0.5% of cases), its phylogenetic tree is presented in **Fig. 6**. No genetic material of Kemerovo virus was detected in the examined ticks.

In certain cases, several tick-borne pathogens were detected in one tick. Thus, DNA of two tick-borne

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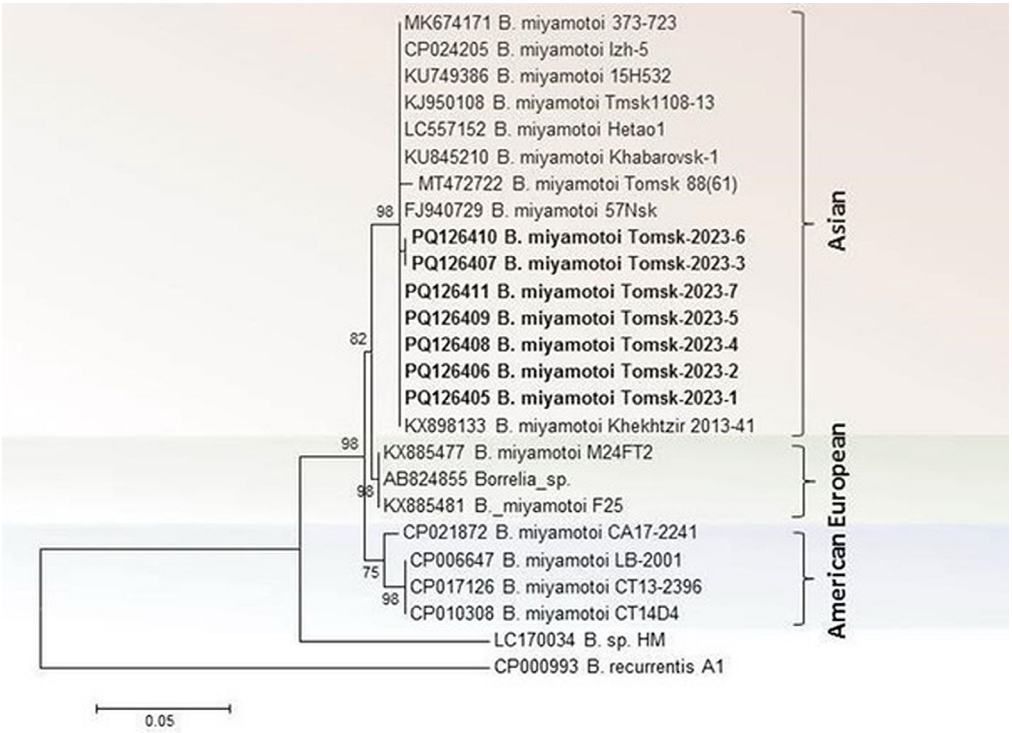

Fig. 4. Phylogenetic tree based on the glpQ gene fragment (433 bp) of the identified *B. miyamotoi* isolates.

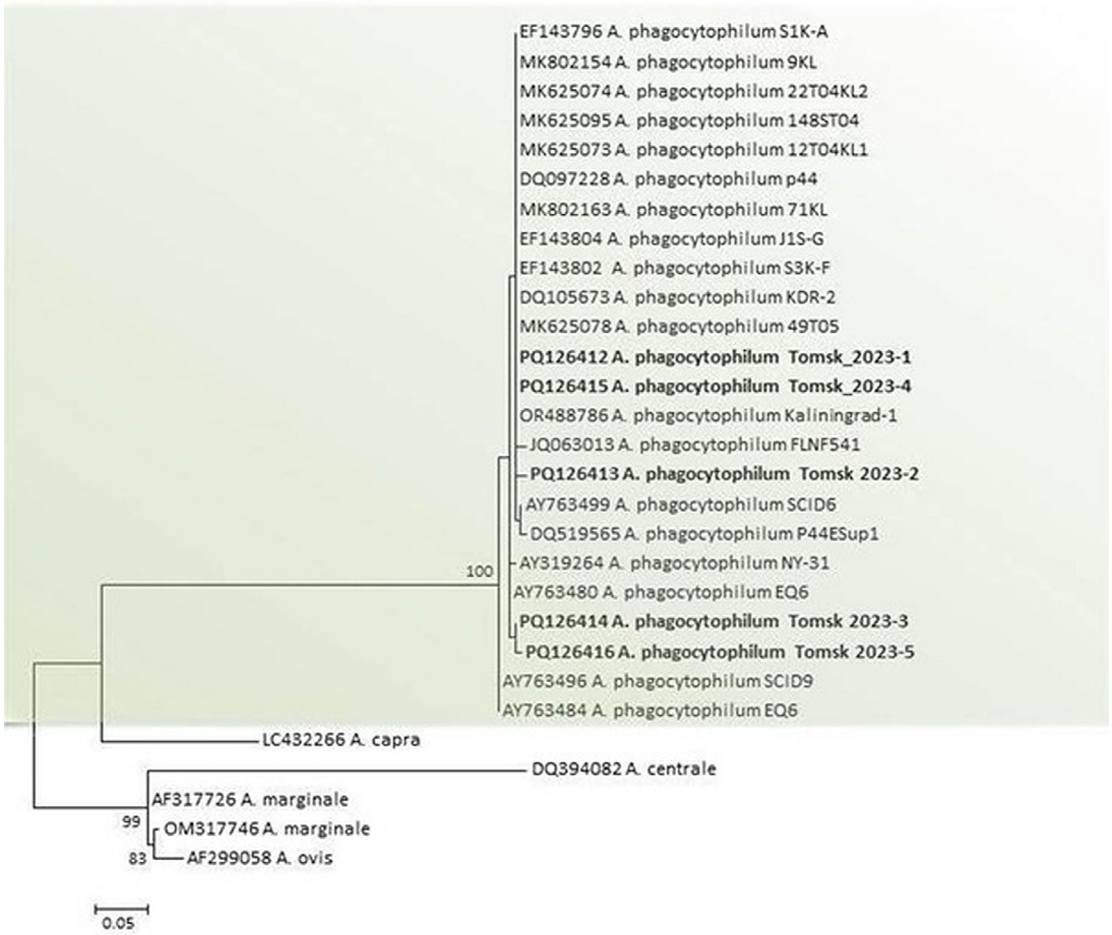

Fig. 5. Phylogenetic tree based on the *msp2* gene fragment (340 bp) of the identified *A. phagocytophilum* isolates.

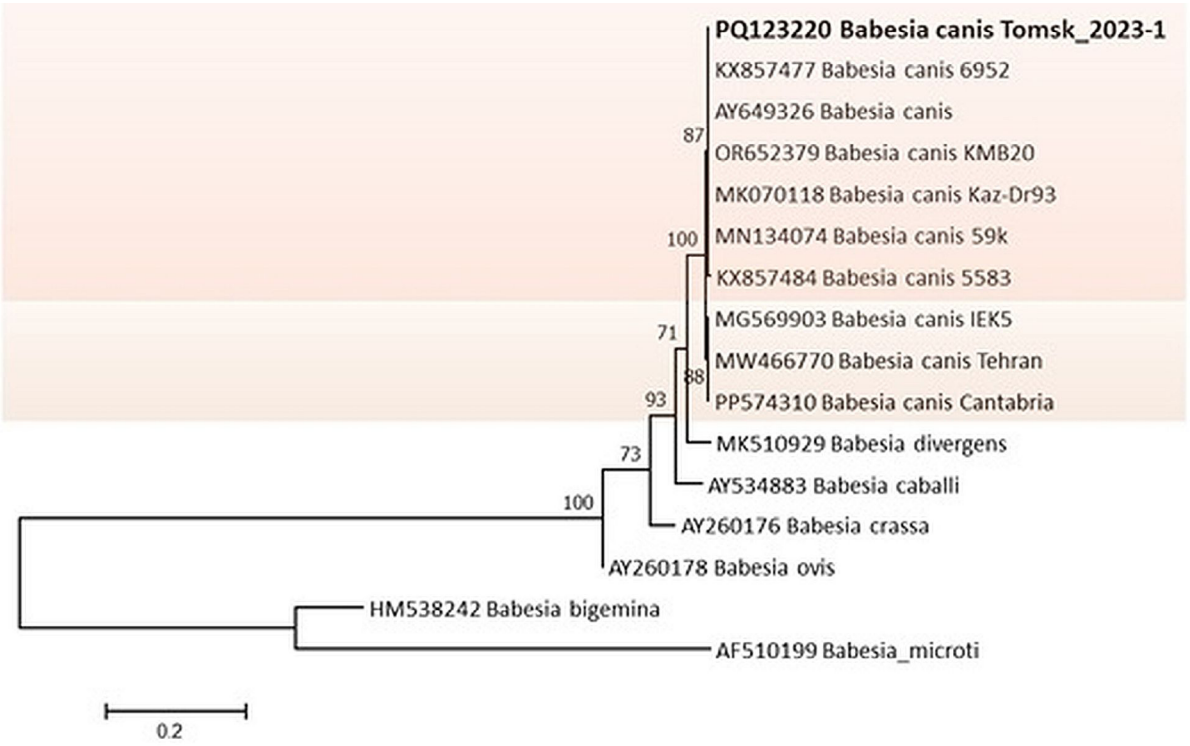

Fig. 6. Phylogenetic tree based on the 18S ribosomal RNA gene fragment (394 bp) of the identified *B. canis* isolate.

pathogens was detected in 8 (2.3%) ticks of the *Ixodes* genus. Genetic material of *B. garinii* and *R. tarasevichiae* was detected in 5 ticks, *B. garinii* and *B. miyamotoi* — in 2 ticks, *B. garinii* and *A. phagocytophilum* — in 1. Moreover, DNA of 3 tick-borne pathogens (*B. garinii, R. tarasevichiae* and *A. phagocytophilum*) was detected in 1 tick of the *Ixodes* genus at once.

Discussion

The *I. pavlovskyi* and *D. reticulatus* species were dominant among the studied ixodid ticks (79.9%), which is recently characteristic of Tomsk and Novosibirsk and their suburbs [6–8]. Thus, the increase in the abundance of *D. reticulatus* more than 200 times in urban biotopes of Tomsk was first recorded in the fall of 2015. When determining the species composition of ticks attacking humans in Novosibirsk and its suburbs, it was also recorded that *I. pavlovskyi* and *D. reticulatus* ticks account for 84.8% among ticks removed from patients seeking medical care for tick bites in 2018. The dominance of *I. pavlovskyi* and *D. reticulatus* ticks in 2023 in the biotopes of Tomsk and its suburbs indicates the stable nature of this phenomenon and the actual displacement of the taiga tick from the biotopes of these metropolitan areas in the south of Western Siberia. At the same time, an independent study in 2019 of a natural biotope on the Tom River upstream of Tomsk (approximately 125 km) revealed a practically complete dominance of the taiga tick, whose representation amounted to 95.72% in collections from vegetation, while the shares of the *I. pavlovskyi* tick and its hybrids amounted to only 1.75 and 2.53%, respectively, with a comparable sample size [19]. Such a dramatic difference in the species composition of ticks in urban and natural biotopes in the basin of one river in the south of Western Siberia suggests that anthropogenic impact significantly changes the breeding conditions of ticks and the spectrum of associated tick-borne infections in places of compact human habitation.

As a result of the studies, genetic material of 9 species of tick-borne pathogens of viral, bacterial and protozoic nature was detected in ixodid ticks of three species. TBEV was detected in 4 ticks (taiga ticks), and all detected TBEV isolates were attributed to the Siberian genotype. It is known that in northern Eurasia TBEV is mainly represented by three main genotypes — Far Eastern, Siberian and European, the first of which most often causes severe clinical forms of TBEV, and the second one is more often found in Western Siberia [5]. The isolates of the Siberian TBEV genotype that we have detected can be attributed to subclade V, for which the Zausaev strain is considered to be the prototype virus. Initially, the Zausaev strain was isolated in Moscow from the brain of a deceased patient with a chronic form of tick-borne encephalitis (within 2 years), who was presumably infected in the Tomsk region and became ill 10 years after a tick bite in 1973. [20].

However, in Tomsk and its suburbs, circulation of TBEV variants of the Siberian subtype in 2006–2008, belonging to subclade IV, strains Kolarovo-2008 (FJ968751) and Tomsk-PT122 (KM019545), as well as variants of the Far Eastern genotype, was previously de-

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tected [7, 9, 21]. Phylogenetic analysis of full-genome sequences showed that isolates of TBEV 2023 are new to Tomsk, with the unusual isolate Tomsk 2023-2 forming a separate phylogenetic branch that may be promising for isolation into a separate subclade within the Siberian TBEV genotype. We failed to detect the genetic material of the Kemerovo virus in the studied ticks, although there are reports of its detection in Western Siberia and Kazakhstan [12].

Infection of ticks of the *Ixodes* genus with *B. burgdorferi s.l.* amounted to 8.5%, which is below the average values of recent years [1, 2, 6, 22, 23]. *B. garinii* was detected most frequently (65.5% of cases), *B. afzelii* (24.1%) and *B. bavariensis* (10.4%) were detected less frequently. The population of borreliae pathogens of ITB is heterogeneous, counting more than 20 species of borreliae, and the species diversity of borreliae is significantly influenced by the diversity of reservoir hosts, which ensure circulation and persistence of ITB pathogens. *B. afzelii* and *B. bavariensis* are commonly associated with small rodents and *B. garinii* with birds [23]. It is likely that the dominance of *B. garinii* can be attributed to the fact that birds may be an important feeder of ixodid ticks in urban areas [1, 8, 13, 24]. *B. burgdorferi s.l.* DNA in the ixodid ticks of the *Dermacentor* genus was unable to be detected, but the possibility of the presence of borrelia in *D. reticulatus* ticks is confirmed by a number of publications [23, 24]. Phylogenetic analysis of sequenced borrelia DNA fragments showed that borrelia isolates formed compact monophyletic groups within their species. Moreover, *B. garinii* isolates were grouped into 3 different phylogenetic branches. At the same time, one of these groups, including 9 isolates, was not associated with isolates previously found in Tomsk. Probably, we can speak about the appearance of new genetic variants of *B. garinii* in urban biotopes of Tomsk. This assumption applies equally to *B. afzelii* and *B. bavariensis* isolates, which also form new phylogenetic groups.

B. miyamotoi DNA was detected in 2.1% of the examined ticks of the *Ixodes* genus. This species of *Borrelia* belongs to the causative agents of tick-borne relapsing fevers, which are widespread in various regions of the world, including Russia [25–28]. This species of borrelia is associated with erythematous forms of ITB, while the pathogen is capable of causing severe forms of disease, including meningoencephalitis, in immunocompromised people, and mixt-infections with other tick-borne pathogens. Infection of ixodid ticks with *B. miyamotoi* is usually much lower than with Borrelia of other species and ranges from 0.3–16% [27, 28]. In 2023, we were unable to detect *B. miyamotoi* DNA in ixodid ticks of the *Dermacentor* genus, although in 2021 in the Tomsk region it was detected in 2% of *D. reticulatus* ticks [3]. Phylogenetic analysis of 7 *B. miyamotoi* isolates based on the *glpQ* gene fragment showed that all of them form a rather compact genetic group within the Asian subtype and cluster together with the previously detected variants in the Tomsk, Novosibirsk Regions, Khabarovsk and Krasnoyarsk Krai.

The highest infection rate of the studied ticks was found for rickettsiae (48.7% for *R. raoultii* and 14.1% for *R. tarasevichiae*), with *R. tarasevichiae* occurring only in ticks of the *Ixodes* genus and *R. raoultii* in ticks of the *Dermacentor* genus. These rickettsiae are capable of causing tick-borne rickettsioses in humans, and their circulation has been established in different regions of the Russian Federation, mainly in Siberia and Kazakhstan [29, 30]. In the Tomsk region, only single cases of rickettsiosis are registered annually [2, 4]. *R. raoultii* is known to occur in many European countries, in different regions of Russia, such as Novosibirsk, Omsk, Irkutsk, in the Republics of Altai and Buryatia, in Primorsky and Khabarovsk Krai and is usually associated with ticks of the *Dermacentor* genus [6, 7, 10, 30]. It is currently accepted that *R. raoultii* is capable of causing the development of TIBOLA syndrome (tick-borne lymphadenopathy), which is characterized by a primary affect in the form of erythema developing at the site of tick sucking and painful regional lymph nodes [31, 32]. Detection of genetic material of *R. raoultii* in meadow ticks, for which an explosive increase in numbers (more than 200 times) in urban biotopes has been registered, requires special attention to the diagnosis of TIBOLA syndrome in patients in the Tomsk region. R. tarasevichiae is characterized by infection of ticks of the *Ixodes* genus (more often *I. persulcatus*, less often *I. pavlovskyi*). This species of rickettsiae is widespread in the Asian part of Russia, and cases of human infection with *R. tarasevichiae* were recorded in the Novosibirsk region [6].

Genetic markers of *A. phagocytophilum* were detected in 1.5% of ticks of the *Ixodes* genus and were not found in ticks of the *Dermacentor* genus, although earlier the genetic material of *A. phagocytophilum* was detected in ticks of *D. reticulatus* in the territory of Tomsk [7]. All sequenced isolates of *A. phagocytophilum* form a monophyletic group within their species with isolates previously detected in Poland, Kaliningrad, and North America, which, in all probability, demonstrates the conservatism of the *msp2* gene used for genotyping. Babesia infections of ixodid ticks were also previously registered in the Tomsk region [2]. As a result of this study, we managed to detect *Babesia canis* in only 1 tick of the *Dermacentor* genus.

Mixed infections associated with various tick-borne infections are quite common and can affect the course and clinical manifestations of diseases [33]. We detected different combinations of tick-borne pathogens in ixodid ticks, with *B. garinii* occurring in all cases of mixed infection.

Comparing the infection rates of different tick species, a significantly higher infection rate of Borrelia was found (OR = 3.1; 95% CI 1.43–6.72; F = 0.004; $\chi^2 = 8.9$), rickettsiae (OR = 18.81; 95% CI 7.25–48.82;

F = 0.000; χ^2 = 60.17) and anaplasmas (OR = 8.75; 95% CI 0.97–79.2; F = 0.038; χ^2 = 5.35) of *I. persulcatus* ticks compared to *I. pavlovskyi*. A wider range of pathogens (TBEV, *B. burgdorferi s.l., B. miyamotoi, R. tarasevichiae* and *A. phagocytophilum*) was recorded in *I. pavlovskyi* ticks which were dominant in urban biotopes. Ticks of the *Dermacentor* genus were predominantly infected with *R. raoultii*, and the other tick-borne pathogens were found in them much less frequently.

The obtained data confirm the necessity of monitoring the circulation in natural and anthropourgical foci of tick-borne infections in Tomsk and Tomsk region along with TBEV and pathogens of other tick-borne infections: *B. miyamotoi*, *Rickettsia* spp., *A. phagocytophilum*, *Babesia* spp. It is necessary to further improve methods of diagnostics and prevention of these infections, including identification of possible human cases and mixtinfection. It is important to emphasize that currently 3 species of ticks (*I. persulcatus, I. pavlovskyi, D. reticulatus*), infected with at least 9 species of tick-borne pathogens, dominate in urban biotopes and take part in the formation of urban foci of tick-borne infections in the park zone of Tomsk.

Conclusion

In the territory of Tomsk and its suburbs, *I. pavlovskyi* and *D. reticulatus* predominate among ixodid ticks collected from vegetation. As a result of PCR analysis, 9 species of tick-borne pathogens of viral, bacterial and protozoan nature were detected in the ixodid ticks of 3 species, which apparently take part in the formation of urban foci of tick-borne infections. Higher levels of infection with borrelia, rickettsiae and anaplasma were found in *I. persulcatus* ticks compared to *I. pavlovskyi*. A wider range of pathogens (TBEV, *B. burgdorferi s.l., B. miyamotoi, R. tarasevichiae* and *A. phagocytophilum*) was recorded in *I. pavlovskyi* and *I. persulcatus* ticks than in *D. reticulatus* ticks (TBEV, *R. raoultii* and *Babesia canis*).

Infection of taiga ticks with TBEV amounted to 1.3%. Infection of ticks of the *Ixodes* genus amounted to the following: *B. burgdorferi s.l.* — 8.5%, *B. miyamotoi* — 2.1%, *A. phagocytophilum* — 1.5%, *R. tarasevichiae* — 14.1%. Furthermore, the rate of occurrence of *R. raoultii* in *D. reticulatus* ticks amounted to 48.7%, and *Babesia canis* DNA was detected in a single sample. Genotyping of tick-borne pathogens was carried out on the basis of sequencing of isolated gene fragments of TBEV, *B. burgdorferi s.l.*, *B. miyamotoi*, *A. phagocytophilum* and *Babesia canis*. All detected TBEV isolates were assigned to the Siberian genotype, subclade V, by analyzing the full-length nucleotide sequence of the genome. The sequences were deposited in GenBank.

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The combined action of *ERG11* gene overexpression and its mutations in the development of *Candida albicans* resistance to triazolic antifungals

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Abstract

Introduction. Modern medicine is faced with the resistance of *Candida* spp. to antimycotics, due to changes in the expression and structure of the *ERG11* gene, the molecular target of triazoles. These mechanisms often operate simultaneously, but the interaction between them remains poorly understood.

The aim of this study is to investigate the interaction between *ERG11* gene overexpression and mutation in the development of triazole resistance in *C. albicans*.

Materials and methods. Eleven *C. albicans* strains from the G.N. Gabrichevsky Moscow Research Institute of Epidemiology culture collection were analyzed. Each strain was characterized by its *ERG11* gene expression level, the presence of *ERG11* mutations, and its susceptibility to the triazoles posaconazole, voriconazole, itraconazole and fluconazole.

Results. The *C. albicans* strains (*n* – number of tested strains) were categorized into four groups: Group 1 (*n* = 2, *ERG11* overexpression only), Group 2 (*n* = 3, ERG11 mutations only), Group 3 (*n* = 4, both *ERG11* overexpression and mutation) and Group 4 (*n* = 2, neither *ERG11* overexpression nor mutation). The minimum inhibitory concentration (MIC) of Triazoles in Group 1 was 15.76-fold higher than in Group 2, 4.97-fold higher than in Group 3, and 2.51-fold lower than in Group 4 ($p < 0.05$ for all comparisons). The MIC of triazoles in Group 2 was 3.17-fold lower than in Group 3 and 40.00-fold lower than in Group 4 ($p < 0.001$). The MIC of triazoles in Group 3 was 12.5-fold lower than in Group 4 ($p < 0.001$). Population-level variation in triazoles MIC was more strongly influenced by the isolated effect of *ERG11* mutations (45.94%) than by the isolated effect of *ERG11* overexpression (5.27-fold less).

Conclusion. Triazole resistance in *C. albicans* is influenced by the combined actions of *ERG11* overexpression and mutation. *ERG11* overexpression appears to contribute more to the absolute level of resistance, while *ERG11* mutations have a greater impact on the diversity of resistance levels within the *C. albicans* population.

Keywords: *Candida albicans, antimycotics, resistance, ERG11 gene, overexpression, mutations*

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ORIGINAL RESEARCHES

Оригинальное исследование
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Сочетанное действие гиперэкспрессии и мутаций гена *ERG11* при формировании резистентности *Candida albicans* к триазоловым противогрибковым препаратам

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Аннотация

Введение. Современная медицина сталкивается с резистентностью *Candida* spp. к антимикотикам, обусловленной изменением экспрессии и структуры гена *ERG11* — молекулярной мишени триазолов. Эти механизмы часто действуют одновременно, однако взаимодействие между ними остаётся недостаточно изученным.

Цель работы — изучение роли гиперэкспрессии гена *ERG11* и его мутаций в формировании резистентности грибов *C. albicans* к триазолам.

Материалы и методы. Исследование выполнено на 11 штаммах грибов *C. albicans* из коллекции МНИИЭМ им. Г.Н. Габричевского. Штаммы были охарактеризованы по уровню экспрессии гена *ERG11* и наличию в нем мутаций, а также чувствительности к триазолам: позаконазолу, вориконазолу, итраконазолу и флуконазолу.

Результаты. Штаммы *C. albicans* подразделили на 4 группы: 1-я группа — только с повышенной экспрессией гена *ERG11*; 2-я — только с мутациями в данном гене; 3-я — одновременно оба вида генетических изменений; 4-я — без данных генетических изменений. Установлено, что минимальная подавляющая концентрация (МПК) триазолов в 1-й группе была в 15,76 раза выше, чем во 2-й, в 4,97 раза выше, чем в 3-й, и в 2,51 раза ниже, чем в 4-й (везде $p < 0,05$). Во 2-й группе МПК триазолов была в 3,17 раза ниже, чем в 3-й, и в 40 раз ниже ($p < 0,001$), чем в 4-й. МПК триазолов в 3-й группе по сравнению с 4-й группой была в 12,5 раза ниже ($p < 0,001$). Популяционное варьирование МПК триазолов в большей степени зависит от изолированного действия мутаций гена *ERG11* (45,94%), что в 5,27 раза превосходит эффект изолированной гиперэкспрессии гена.

Заключение. Устойчивость *C. albicans* к триазолам обеспечивается кооперативным действием гиперэкспрессии и мутаций гена *ERG11*: наибольшую резистентность обеспечивает гиперэкспрессия, популяционное разнообразие — мутации.

Ключевые слова: *Candida albicans, антимикотики, резистентность, ген ERG11, гиперэкспрессия, мутации*

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

Microbial resistance to chemotherapeutic drugs is a longstanding challenge in modern medicine. While numerous mechanisms of antibiotic resistance are well-characterized, including those genetically encoded that increase antibiotic target production or alter target structure, the interplay of these mechanisms remains poorly understood [1–3]. These resistance mechanisms can operate independently or concurrently within a microbial cell, and the consequences of their combined effects require further investigation.

We investigated this issue using *Candida* species as a model, given their well-documented resistance to antimicrobial drugs. One resistance mechanism involves increased expression of genes encoding drug targets, notably *ERG11*, which encodes lanosterol 14α-demethylase. This enzyme is crucial for ergosterol biosynthesis, a key component of the fungal cell wall. *ERG11* overexpression leads to increased ergosterol production, rendering *Candida* species insensitive to therapeutic azole concentrations [4].

However, recent studies have identified non-synonymous *ERG11* mutations that modulate its effects, impacting Candida's triazole susceptibility both positively and negatively [5–9]. Our data [10] show that certain *ERG11* mutations mitigated the effects of overexpression, reducing the Minimal Inhibitory Concentration (MIC) of triazole drugs in mutant Candida albicans strains by up to 100-fold. Complete reversal of resistance, however, was not observed. It is important to note that *ERG11* overexpression and mutations appear to manifest relatively independently across different *Candida* spp. [5, 7–9, 11–15].

Both *ERG11* overexpression and mutation clearly contribute to the population-level diversity in azole sensitivity observed in Candida species. However, the precise nature and outcome of the interaction between these mechanisms remain unclear. Investigating this interaction is crucial for understanding the survival strategies employed by *Candida* spp. under conditions of widespread drug exposure and may reveal promising avenues for combating the growing problem of antimicrobial resistance.

Therefore, **the aim** of this study was to investigate the interaction between *ERG11* overexpression and mutation in the development of triazole resistance in *C. albicans*.

Materials and methods

The study was conducted on 11 *C. albicans* strains from the collection of the G.N. Gabrichevsky Moscow Research Institute of Epidemiology and Microbiology (Rospotrebnadzor), which were initially resistant to the effects of fluconazole and voriconazole.

Strain identification was performed using biochemical assays and real-time multiplex polymerase chain reaction (qPCR), along with *ERG11* expression level analysis and mutation screening. A detailed description of the methodology is provided in another study [10].

According to the available characterization, 7 of the studied strains were carriers of 5 variants of non-synonymous mutations in the *ERG11* gene (*E266D*, *G464S*, *I471L*, *D116E*, and *V488I*), while 6 strains showed *ERG11* overexpression.

Based on these genetic characteristics, *C. albicans* strains were divided into 4 groups: Group 1 (n=2)—strains with only *ERG11* overexpression; Group 2 (n = 3) — strains with only *ERG11* mutations; Group 3 (n = 4) — strains with simultaneous expression of both types of genetic alterations; Group 4 (n = 2) — strains without either of the genetic alterations.

The sensitivity of the studied *C. albicans* strains to four representatives of triazole antifungals (posaconazole, voriconazole, itraconazole, fluconazole) was investigated in accordance with the recommendations of the Interregional Association for Clinical Microbiology and Antimicrobial Chemotherapy (IACMAC) for determining the sensitivity of microorganisms to antimicrobial agents, based on CLSI M44 and M60 standards for fungi and the standards and criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for microdilution methods and bacterial cultures[1].

The minimum inhibitory concentrations (MIC, mg/mL) were determined by the serial microdilution method using the Sensititre YeastOne plates (Trek diagnostic system). For this, the inoculum was prepared similarly to the disk diffusion method, after which it was introduced into a modified RPMI-1640 medium and distributed into 96-well plates for serial microdilutions with previously added triazole antifungals [11]. The results were recorded visually, comparing the growth in the well with the positive control well according to EUCAST criteria [12].

To ensure the comparability of the research results, the data for individual *C. albicans* strains for each triazole antifungal were weighted by the average MIC value for the given drug. Subsequently, the obtained relative values were analyzed.

Statistical analyses were conducted using Microsoft Excel, SciPy and Matplotlib. The significance of the differences was assessed using the Mann–Whitney U-test. The contribution of factors to the population variability of the trait was assessed using single-factor and two-factor ANOVA. The critical error level for testing statistical hypotheses was set at $p < 0.05$.

Results

The MIC of triazole antifungals with various genetic modifications in *C. albicans* is presented in **Table 1**.

[1] IACMAC Recommendations "Determination of the sensitivity of microorganisms to antimicrobial drugs (2021)». URL: https://www.antibiotic.ru/minzdrav/category/clinical-recommendations

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Table 1. MIC of triazole antifungals in various genetic modifications in the *ERG11* gene of *C. albicans* ($X \pm m$)

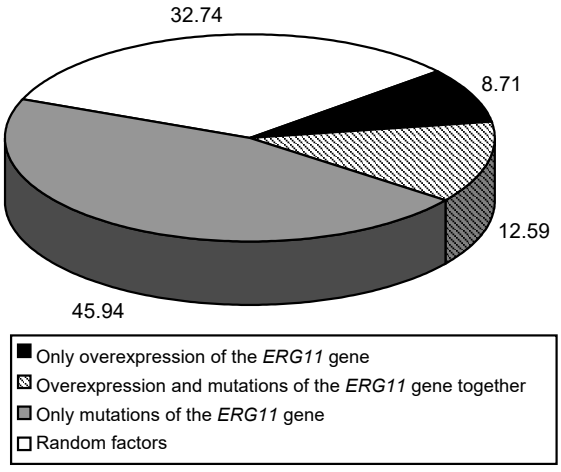
Strain group	*n*	Posaconazole	Voriconazole	Itraconazole	Fluconazole
1	2	1.361 ± 1.351	1.184 ± 1.045	1.363 ± 1.353	1.579 ± 1.483
2	3	0.008 ± 0.002	0.139 ± 0.000	0.008 ± 0.002	0.191 ± 0.000
3	4	0.028 ± 0.019	0.383 ± 0.244	0.026 ± 0.020	0.669 ± 0.317
4	2	4.068 ± 1.357	3.343 ± 1.115	4.075 ± 1.359	2.296 ± 0.765

Statistical analysis revealed no significant differences between the individual drugs for each variant of genetic alterations, indicating a uniform directional effect across all triazoles. Due to this fact, the results of the MIC study were pooled into a single group of triazoles. The defining characteristics of each group are presented in **Table 2**.

Comparative analysis of the obtained results showed that the MIC of triazoles in Group 1 was 15.76 times higher ($p < 0.05$) than in Group 2, 4.97 times higher ($p < 0.05$) than in Group 3, and 2.51 times lower ($p < 0.05$) than in Group 4. In Group 2, the MIC of triazoles was 3.17 times lower than in Group 3, and 40 times lower ($p < 0.001$) than in Group 4. The MIC of triazoles in Group 3 was 12.5 times lower ($p < 0.001$) compared to Group 4.

The assessment of the impact of various genetic alterations on the degree of variation in the MIC of triazoles in the studied *C. albicans* population was conducted using analysis of variance (ANOVA). The single-factor model showed that the combined effect of *ERG11* overexpression and mutation amounts to 58.58% ($p < 0.001$) of the total variance.

A two-factor ANOVA was performed to quantify the relative impact of these genetic alterations (**Figure**). The isolated effect of *ERG11* mutations accounted for almost half (45.94%) of the genetic variance, which is more than 5.27 times greater than the contribution of the isolated effect of *ERG11* overexpression and 3.65 times greater than the combined effect of mutations and overexpression. Taken together, the combined effect of all genetic alterations accounts for 67.26%, which is consistent with the findings of the single-factor ANOVA model.



Two-factor model of the influence of genetic alterations in the *ERG11* gene on the variation of triazole MIC in the *C. albicans* population, %.

Table 2. MIC of triazole antifungals in the studied groups

Strain group	*n*	$X \pm m$	Me [Q_1; Q_3]
1	8	1.371 ± 0.501	1.184 [0.010; 2.470]
2	12	0.087 ± 0.024	0.075 [0.007; 0.139]
3	16	0.276 ± 0.113	0.112 [0.006; 0.152]
4	8	3.445 ± 0.522	2.889 [1.879; 3.759]

Discussion

This study confirms that both *ERG11* overexpression and *ERG11* mutations contribute to triazole resistance in *C. albicans* strains initially resistant to fluconazole and voriconazole. It is further demonstrated that these genetic mechanisms can act independently or synergistically in conferring resistance. While *ERG11* overexpression generally exerts a more pronounced effect than *ERG11* mutations alone, as confirmed in several previous studies [4-9, 16, 17], their interaction is complex.

Although an additive effect of *ERG11* overexpression and mutations might be anticipated, our data reveal that certain mutations can attenuate the impact of *ERG11* overexpression. This resulted in a noticeable reduction in the overall effect of *ERG11* overexpression in our *C. albicans* strain collection. However, it is not recommended to generalize this observation to all instances of genetically mediated resistance in *C. albicans*; rather, this finding is interpreted as a potential characteristic specific to the strains included in this study.

The observation of high triazole resistance in *C. albicans* strains lacking *ERG11* alterations suggests that other resistance mechanisms are also operative. For example, overexpression of efflux pump genes, such as *CDR1*, *CDR2* and *MDR1*, has been reported [4, 5], although their relative contributions to resistance remain to be fully quantified.

The analysis of variance accounted for the contribution of both *ERG11* overexpression and mutation to the population-level variation in triazole susceptibility among *C. albicans* strains. While both mechanisms

contribute, the results indicate that *ERG11* mutations play a dominant role in shaping this phenotypic diversity.

Evaluating the biological and medical significance of the overexpression and mutations of the *ERG11* gene in *C. albicans* strains, it was observed that *ERG11* overexpression and the associated lanosterol-14α-demethylase hyperproduction serve as a far more effective defense mechanism against the harmful effects of triazole antifungals than the synthesis of genetically modified enzyme variants. However, non-synonymous point mutations in *ERG11* clearly contribute to the increased biological diversity of this yeast-like fungus, without necessarily causing a dramatic, short-term increase in its clinical threat. Therefore, from a practical perspective, identifying *ERG11* overexpression may be a more appropriate initial strategy for predicting the immediate risk of triazole resistance in *C. albicans* isolates.

Conclusion

1. Triazole resistance in *C. albicans* strains arises from the combined effects of *ERG11* overexpression and mutation.

2. *ERG11* overexpression has a significantly greater impact on resistance levels than its non-synonymous mutations.

3. Mutations within the *ERG11* gene are a more significant driver of population-level triazole resistance diversity in *C. albicans* than *ERG11* overexpression.

4. It is recommended to test strains for *ERG11* overexpression to predict the emergence of triazole resistance in *C. albicans*.

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Global Forecasting Models for Dengue Outbreaks in Endemic Regions: A Systematic Review

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Abstract

Background. Dengue is a rapidly spreading mosquito-borne disease, posing significant global health challenges, particularly in endemic regions. Recent years have witnessed an increase in the frequency and intensity of dengue outbreaks, necessitating robust forecasting models for early intervention.

This systematic review **aims** to synthesize recent literature on dengue forecasting models, evaluate their predictive performance, and identify the most effective approaches.

Materials and methods. A comprehensive search in Scopus, PubMed, ScienceDirect, and Springer databases was conducted following PRISMA guidelines. Studies were selected based on strict inclusion and exclusion criteria, and the quality of the research was evaluated using TRIPOD criteria. Out of 1,366 identified studies, 13 met the eligibility criteria. Data were extracted and analyzed to assess the accuracy and validity of the forecasting models employed.

Results. The findings indicate that machine learning-based models, particularly random forest, outperform conventional statistical models such as ARIMA and Poisson regression. Additionally, climate data — especially temperature and rainfall play a critical role in forecasting dengue incidence.

Conclusion. The present study corroborates the superior efficacy of machine learning-based forecasting models, particularly random forest, in forecasting dengue cases compared to conventional statistical methods. This finding provides a foundation for the development of an enhanced early warning system to address future outbreaks of dengue.

Keywords: *dengue, forecast model, machine learning, random forest, early warning system*

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Оригинальное исследование
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Модели глобального прогнозирования вспышек денге в эндемичных регионах: систематический обзор

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Аннотация

Введение. Лихорадка денге — быстро распространяющееся заболевание, переносимое комарами, представляет серьёзную проблему для глобального здравоохранения, особенно в эндемичных регионах. Частота и интенсивность вспышек лихорадки денге увеличиваются, что требует создания надёжных моделей прогнозирования для раннего вмешательства.

Цель систематического обзора — обобщить данные литературы о моделях прогнозирования лихорадки денге, оценить их прогностическую эффективность и выявить наиболее эффективные подходы.

Материалы и методы. Всесторонний поиск в базах данных Scopus, PubMed, ScienceDirect и Springer проведён в соответствии с рекомендациями PRISMA. Исследования отбирали на основе строгих критериев включения и исключения, а качество исследований оценивали с помощью критериев TRIPOD. Из 1366 выявленных исследований 13 соответствовали критериям отбора. Данные были проанализированы для оценки точности и обоснованности использованных моделей прогнозирования.

Результаты. Результаты показывают, что модели на основе машинного обучения, в частности «случайный лес», превосходят традиционные статистические модели, такие как ARIMA и регрессия Пуассона. Кроме того, климатические данные, особенно температура и количество осадков, играют важную роль в прогнозировании заболеваемости лихорадкой денге.

Заключение. Настоящее исследование подтверждает более высокую эффективность моделей прогнозирования на основе машинного обучения, в частности «случайный лес», в прогнозировании случаев заболевания лихорадкой денге по сравнению с традиционными статистическими методами. Этот вывод закладывает основу для разработки усовершенствованной системы раннего оповещения для борьбы с будущими вспышками лихорадки денге.

Ключевые слова: *денге, модель прогнозирования, машинное обучение, метод случайного леса, система раннего оповещения*

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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 one of the fastest spreading mosquito-borne disease, especially in tropical and subtropical regions, caused by various types of dengue viruses [1, 2]. The World Health Organization has reported an 8-fold increase in global dengue incidence between 2000 and 2019. In 2023, over 5 million cases were documented across 80 countries, with at least 23 nations experiencing dengue outbreaks. That number has more than doubled in 2024, with more than 10.6 million cases reported in North and South America alone. However, the actual number of cases is likely significantly higher, emphasizing the urgent need for effective public health interventions to mitigate this escalating crisis [3]. Although most infections are harmless, dengue shock syndrome and dengue are severe forms of infection that can lead to death [4, 5]. In the absence of a specific drug or vaccine for this virus, case fatality rates can reach 20% if diagnosis is not prompt [6], particularly in resource-constrained areas. When outbreaks occur on a large scale, the sheer number of severe dengue cases can overwhelm the health system and impede the delivery of optimal care. Dengue also poses a huge social and economic burden to many tropical countries where the disease is endemic [7]. Precise prediction of outbreak size and trends in disease incidence early can limit further spread [8], and help better plan health resource allocation to meet needs during an outbreak.

The two principal vectors are *Aedes aegypti* and *A. albopictus*, which are capable of transmitting dengue. The transmission of dengue is influenced by a number of factors, including environmental and climate change, urbanization, globalization, vector activity, and behavioral change [9]. The interaction between humans, climate, and mosquitoes gives rise to a complex system that exerts a profound influence on dengue transmission patterns, which in turn affects the likelihood of outbreaks [10]. This relationship has been researched for decades through the development of forecasting models in different parts of the world. These models vary widely, both in terms of purpose [11, 12], and setting [13–15]. While many of these models demonstrate excellence in various tasks, to create efficient prediction models, a systematic, adaptive and generalizable framework is needed, capable of identifying weather- and population-related patterns of vulnerability across geographic regions. The scientific community has not yet reached agreement on which models provide the best predictions. There are many research reports on prediction tools for dengue outbreaks [16–19]. However, research

that provides a comprehensive summary of the performance and predictive ability of these tools remains limited. Previous studies have underscored the value of integrating diverse epidemiological tools, including mapping and mathematical models, to develop an effective early warning system [20]. However, this study did not prioritize the identification of significant predictors in the development of an early warning system for dengue. Other studies that emphasize early warning systems and incorporate numerous case forecasting models have been conducted, but this study solely examines the case experience of the various models utilized [21].

Various forecasting models have been developed over the years, integrating epidemiological, environmental, and climatic variables. While some models rely on traditional statistical methods such as Autoregressive Integrated Moving Average (ARIMA) and Poisson regression [14, 22–24]. Emerging research highlights the superior accuracy of machine learning models, particularly random forest and Long Short Term Memory (LSTM) [25, 26]. However, there is still no consensus on the most effective forecasting approach. To address this research gap, several recent studies have explored novel methodologies in dengue forecasting. Recent studies indicate that integrating deep learning techniques, such as LSTM and transformer models, significantly improves prediction accuracy compared to conventional statistical models [27]. Furthermore, recent findings suggest that incorporating real-time meteorological and mobility data improves forecasting precision [28]. These updated approaches not only improve prediction accuracy but also enhance model adaptability across different geographical regions. Despite these advancements, inconsistencies in data quality, limited external validation, and computational constraints continue to pose challenges in real-world applications. This review focuses on determining which model exhibits the highest accuracy and examining its internal and external validity. Its objective is to synthesize recent literature on dengue case forecasting, discuss related evidence, and evaluate different models' forecasting performance to identify the most effective one.

Materials and methods

This review used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) approach, which includes methods for determining resources, eligibility, inclusion and exclusion criteria, and the process of systematic review, extraction, and analysis of data from the available literature [7]. PRISMA 2020 replaces the previous edition published in 2009, introducing new reporting guidelines that include more comprehensive study identification, selection, scoring, and synthesis methods [29]. This guide enables the search for terms relevant to the review and provides advice on aspects that need to be addressed in the review report for publication purposes [21].

Research Question Formulation

Research questions were developed using PICo, a useful tool to help frame relevant research questions for systematic reviews. The PICo concept incorporates three important elements (population or problem, importance, and context) [30]. Based on PICo, the three main components in this review are dengue (Problem), case forecast model (Importance), and case prediction (Context). These concepts guided the formulation of the research question: "What is the evidence of the dengue case forecast model and its performance in predicting cases?"

Systematic Searching Strategies

Systematic searching strategies include identification, screening, and eligibility process.

Identification

In the identification stage, synonyms and variations were used to enrich the keywords, then applied in the search process, search strings were created and generated by using Boolean operators and keyword search, as illustrated in **Table 1**. A systematic literature search was conducted against four major databases: Scopus, PubMed, ScienceDirect, and Springer, and identified a total of 1366 relevant records. 16 duplicate records were found and removed, leaving 1,350 records for title screening. All potential records were then exported from the databases and organized into Excel sheets for title and abstract screening.

Screening

Two authors were responsible for the screening of titles and abstracts, which was conducted in accordance with the review questions that had been developed and the specific inclusion and exclusion criteria that had been established. Inclusion criteria were primary research in peer-reviewed journals and English-language articles. We excluded systematic review articles, books, conference proceedings, and non-peer-reviewed articles, such as editorials, commentaries, opinion pieces, or short reports. The screening process resulted in the elimination of 1,120 articles that were deemed irrelevant to the review. The remaining 230 articles were then read in full, including the abstracr reading, and assessed for eligibility.

Eligibility

A total of 64 full-text articles were retrieved for eligibility. Two authors independently reviewed all full-text articles for eligibility. All studies found to be unrelated to the interest and outcome of interest were excluded. The reasons for article exclusion were notated. There were 51 articles excluded due to:

1) studies that did not focus on predicting the number of future cases (n = 14);

2) studies that used or evaluated prediction or forecasting models, including machine learning meth-

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Table 1. Keywords search used in the screening process

Databases	Keywords used
Pubmed	((((((((((((((((((((((dengue fever) OR (dengue incidence)) OR (dengue outbreaks)) OR (dengue epidemic)) AND (forecasting models)) OR (predictive models)) OR (prediction models)) OR (epidemic forecasting)) OR (outbreak prediction)) AND (machine learning)) OR (statistical models)) OR (ARIMA)) OR (regression models)) OR (random forest)) OR (neural networks)) OR (support vector machines)) AND (environmental factors)) OR (climate variables)) OR (temperature)) OR (rainfall)) OR (humidity)) OR (climate data)) OR (weather patterns)) AND (endemic regions)) AND (tropical areas)
Scopus	TITLE-ABS-KEY ("dengue fever" OR "dengue incidence" OR "dengue outbreak*" OR "dengue epidemic*") AND ("forecast* model*" OR "predict* model*" OR "prediction model*" OR "epidemic forecast*" OR "outbreak prediction") AND ("machine learning" OR "statistical model*" OR "ARIMA" OR "regression model*" OR "random forest" OR "neural network*" OR "support vector machine*") AND ("environment* factor*" OR "climate variable*" OR "temperature" OR "rainfall" OR "humidity" OR "climate data" OR "weather pattern*") AND ("endemic region*" OR "tropical area*" OR "high-risk area*" OR "disease-endemic region*")
ScienceDirect	Search 1: ("dengue fever" OR "dengue incidence") AND ("forecasting models" OR "prediction models") Search 2: ("dengue fever" OR "dengue incidence") AND ("prediction models" OR "outbreak prediction") AND ("machine learning" OR "statistical models") Search 3: ("dengue fever" OR "dengue outbreaks") AND ("predictive models" OR "forecasting models") AND ("environmental factors" OR "temperature" OR "rainfall")
Springer	("dengue fever" OR "dengue incidence" OR "dengue outbreaks") AND ("forecasting models" OR "predictive models") AND ("machine learning" OR "statistical models" OR "ARIMA") AND ("environmental factors" OR "climate" OR "rainfall") AND ("endemic regions" OR "tropical areas")

ods (random forests, LSTM) or statistical models (such as ARIMA, Seasonal Autoregressive Integrated Moving Average (SARIMA), regression) (n = 19);

3) articles that did not involve key climate variables in the forecasting (n = 11);

4) studies conducted in non-endemic or low prevalence dengue areas (n = 7).

The remaining 13 eligible articles were continued for the quality assessment process.

Quality Assessment

The quality of the study was assessed using the quality assessment criteria described in TRIPOD (Transparent Reporting of multivariable prediction models for Individual Prognosis or Diagnosis) [31]. The TRIPOD statement is a checklist of 22 items, which are considered essential for the proper reporting of research that develops or validates multivariable prediction models [32]. The TRIPOD guidelines explicitly cover the development and validation of prediction models for diagnosis and prognosis across all medical domains and predictor types. Two authors conducted the quality assessment independently. Scores for report levels were obtained by awarding one point for each reported item relevant to the study. The total score was converted to a percentage based on the maximum possible score. Ultimately, 17 articles (with a percentage score > 70%) were included in the review [21]. **Table 2** presents the scores and percentages of each quality assessment adapted from the TRIPOD checklist.

Data Extraction and Synthesis

The author extracted the data independently using a standardized data extraction form and organized it in a Microsoft Excel worksheet. The information collected included: author (year), country, study design, candidate predictors, research, data frequency, model techniques used, model performance, outcome, model accuracy, evaluation. The PRISMA flowchart is shown in **Figure 1**.

Results

Study characteristics

A total of 13 studies met the eligibility criteria and were included in this systematic review. Of these 13 studies, 4 (31%) were conducted in the Americas, 4 (31%) in East Asia, 4 (31%) in Southeast Asia, and 1 (7%) in South Asia. Brazil was the country with the highest number of eligible studies (n = 4) [25, 26, 33, 34], followed by China (n = 2) [27, 35], Taiwan (n = 2) [36, 37], Vietnam (n = 2) [28, 38]. Other studies were conducted in Malaysia [39], Sri Lanka [40], and the Philippines [41]. Five (42%) studies were published between 2015 and 2020, 9 studies between 2018–2022, and 7 (58%) studies were published between 2021–2024. Most studies (46%) used weekly time units, there were 23% studies using monthly data units, and the rest using annual and yearly. More than half (n = 7; 54%) of the studies used machine learning model techniques [25–28, 33, 36, 39], and the remaining (n = 5; 46%) studies used statistical model techniques [34, 35, 37, 38, 40, 41]. The characteristics of the included studies are summarized in **Fig. 2**. Details of the characteristics within each study are presented in **Table 3**.

Approach and Accuracy of Forecasting Model for dengue cases

Various modeling approaches, such as machine learning and statistical methods for dengue case experience have been used in all included studies. Out of 13 studies, 6 (26,1%) used random forest approach

Table 2. Quality appraisal score of eligible articles adapted from TRIPOD checklist [32, 42]

Daftar periksa	Item	Source												
		[25]	[26]	[27]	[28]	[33]	[34]	[35]	[36]	[37]	[38]	[39]	[40]	[41]
Title and abstract														
Title	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Abstract	2	1	1	1	1	1	1	1	1	1	1	1	1	1
Introduction														
Background and objectives	3a	1	1	1	1	1	1	1	1	1	1	1	1	1
	3b	1	1	1	1	1	1	1	1	1	1	1	1	1
Methods														
Source of data	4a	1	1	1	1	1	1	1	1	1	1	1	1	1
	4b	1	0	0	0	1	0	0	1	0	0	0	0	0
Participants	5a	1	1	1	1	1	1	1	1	1	1	1	1	1
	5b	1	1	1	1	1	1	1	1	1	1	1	1	1
Outcome	6a	1	1	1	1	1	1	1	1	1	1	1	1	1
Predictors	7a	1	1	1	1	1	1	1	1	1	1	1	1	1
Sample size	8	1	0	0	0	1	0	0	1	0	0	0	0	0
Missing data	9	1	0	0	0	0	0	0	0	0	0	0	0	0
Statistical analysis methods	10a	1	1	1	1	1	1	1	1	1	1	1	1	1
	10b	1	1	1	1	1	1	1	1	1	1	1	1	1
	10d	1	1	1	1	1	1	1	1	1	1	1	1	1
Results														
Participants	13a	1	0	0	0	1	0	0	0	0	0	0	0	0
	13b	1	1	1	1	1	1	1	1	1	1	1	1	1
Model development	14a	1	1	1	1	1	1	1	1	1	1	1	1	1
	14b	1	0	0	0	0	0	0	1	0	0	0	0	0
Model specification	15a	1	1	0	0	1	1	1	1	1	0	1	1	1
	15b	1	1	1	1	1	1	1	1	1	1	1	1	1
Model performance	16	1	1	1	1	1	1	1	1	1	1	1	1	1
Discussion														
Limitations	18	1	1	1	1	1	1	1	1	1	1	1	1	1
Interpretation	19b	1	1	1	1	1	1	1	1	1	1	1	1	1
Implications	20	1	1	1	1	1	1	1	1	1	1	1	1	1
Other information														
Supplementary information	21	1	0	0	0	0	0	0	1	0	0	0	0	0
Funding	22	1	0	1	1	1	0	1	1	0	1	0	0	0
Final score		27	20	20	20	24	20	21	25	20	20	20	20	20
Percentage		100	74.1	74.1	74.1	88.9	74.1	77.8	92.6	74.1	74.1	74.1	74.1	74.1

[25–27, 33, 36, 39], 5 (21,7%) used LSTM approach [26, 28, 34, 38, 41], 3 (13%) used ARIMA [34, 40, 41], 2 others used Least Absolute Shrinkage and Selection Operator (LASSO), Gradient Boosting, XGBoost poisson regression, SARIMA. In terms of perfomance, all studies use different methods, including Root Mean Squared Error (RMSE), R-Squared (R^2), Pearson Correlation, Mean Absolute Percentage Error (MAPE), RMSE, Mean Absolute Error (MAE), Area Under the Curve (AUC), Mean Squared Error (MSE), Mean Squared Logarithmic Error (MSLE), Akaike Information Criterion (AIC). The type of model used can be seen in **Fig. 3**.

Of the 13 articles included, there are 3 best forecasting methods with the highest model accuracy, namely random forest, LSTM, and LASSO. 6 articles using the random forest method, showed an average model accuracy of 89% [25–27, 33, 36, 39], from 5 articles using the LSTM method, there are 3 articles that show model accuracy, and the average obtained is 89% [26, 28, 38],

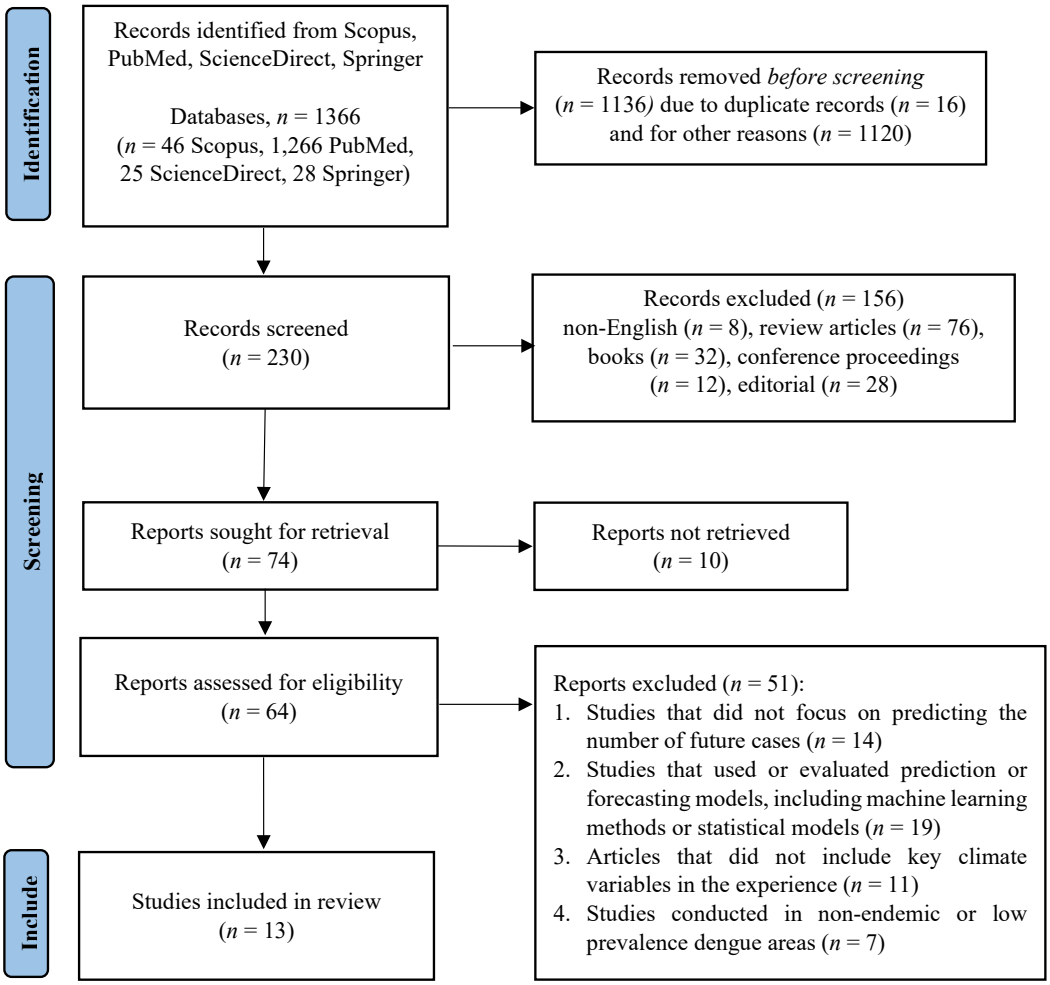

Fig. 1. Systematic review flow.

while the other 2 articles do not mention the percentage of model accuracy [34, 41]. Of the 2 articles that used the LASSO method, the average model accuracy was 65% [26, 33]. The accuracy of the forecasting models can be seen in **Fig. 4**. In general, all of the case experience models included in the study showed fairly good forecasting ability. Overall, climate indicators were the most frequently used in showing the best performance. However, there are studies that used a combination of climate and epidemiological indicators, which showed that previous dengue cases significantly influenced current dengue cases [39].

Random forest model accuracy

The **Figure 5** illustrates the accuracy of various random forest models applied in dengue forecasting studies. The dataset includes models developed by six original research, with accuracy values ranging from 83% to 92%. The average model accuracy is recorded at 89%. The results highlight the superior predictive performance of random forest models in dengue incidence forecasting, reinforcing their potential for integration into early warning systems for outbreak management.

Discussion

This systematic review aims to summarize and discuss the evidence of various dengue case forecasting methods, model performance, and their ability to explain dengue incidence. This review shows that dengue prediction studies have become a topic of research interest, especially in Asia, where 69% of these included studies were conducted in Asia. This trend is due to the fact that the Asian region represents about 70% of the dengue burden globally [43]. Climate data, particularly temperature, rainfall and humidity are important predictors of dengue incidence, but they are often not available in time for health providers working on dengue early warning systems. Several studies have found that countries with better meteorological records provide higher performance metrics [25, 34, 35]. Therefore, integration with local meteorological departments on real-time meteorological data will improve access to meteorological information and benefit end users in early outbreak detection.

In general, climatic variables show an important role in the prediction of dengue cases. Climate variables such as mean temperature [25, 27, 28, 38, 39],

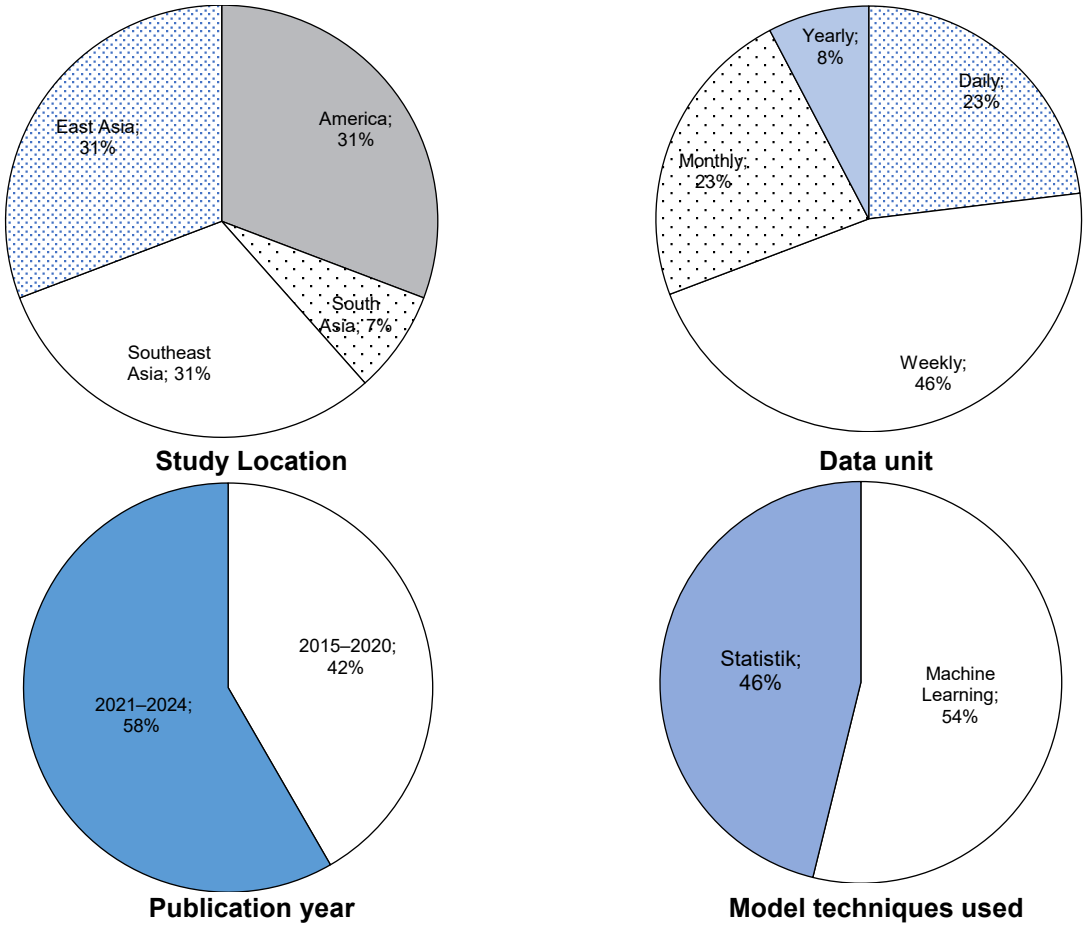

Fig. 2. Study characteristics.

minimum temperature [27, 35–37], maximum temperature [27, 37, 38], rainfall [27, 28, 36, 37, 39], humidity [25, 33, 39, 40], relative humidity [25, 28, 33], wind speed [25, 28, 33], evaporation and sunshine [28] are important input paramaters in the development of dengue incidence prediction models. Temperature showed the best predictive capacity of the meteorological variables studied in this review. In Vietnam, temperature was a significant predictor in the best dengue forecasting model, where the AUC and sensitivity were 87.42%

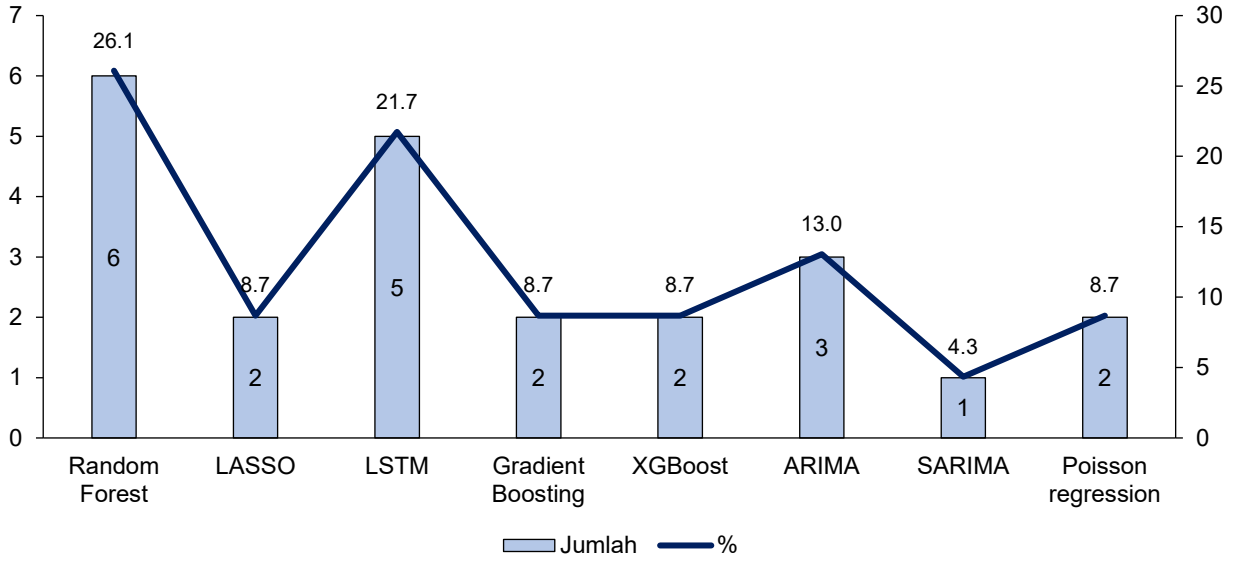

Fig. 3. Type of model technique used.

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Table 3. The details for characteristic and main findings of each study

Source	Country	Study Design	Candidate predictors	Data Unit	Model techniques used	Model performance	Outcome	Model Accuracy	Evaluation
[25]	Brazil	Observational Study	Rainfall, maximum temperature, minimum temperature, relative median temperature, insolation, rate of evaporation, median relative humidity, median wind speed	Monthly	Machine Learning (Random Forests, Gradient Boosting, Multilayer Perceptron, Support Vector Regression)	RMSE, MAE (Lowest errors with Random Forests)	Monthly cases of dengue	RMSE: 15.5 = 84.5% MAE: 11.9 = 88,1%	Internal and External
[26]	Brazil	Comparative Study	Historical dengue cases, climate variables, tweets	Weekly	Machine Learning (LSTM, Random Forest, LASSO)	MSE, MSLE	Forecasting dengue incidence	LSTM = MSE = 0,04 (96%), MSLE = 0,01 (100%) Random Forest = MSE = 0,17 (83%), MSLE = 0,13 (87%) LASSO = MSE = 0,4 (60%), MSLE = 0,33 (67%)	Internal and external
[27]	China	Spatiotemporal Analysis	Imported cases, Tmin, Forest, Pop, Prec, Tmean, GDP, RH, Cropland, Tmax, Impervious, Water	Daily	Random Forest, Gradient Boosting Machine, Support Vector Machine	AUC	Dengue incidence	AUC = 0.91 (91%)	Internal and external
[28]	Vietnam	Observational	Climate data (temperature, precipitation, humidity, evaporation, sunshine hours)	Daily	Machine Learning (LSTM, LSTM-ATT, CNN, Transformer)	RMSE and MAE	Forecasting dengue fever incidence	RMSE: 1.60 MAE: 1.95 Accuraty rate 100%	Internal only
[33]	Brazil	Quantitative research design	Epidemiological data, Google search data, Weather	Weekly	Random Forest, LASSO Regression	RMSE, R^2, Pearson Correlation	Dengue incidence	LASSO = 70%-90% Up to 90%	Internal only
[34]	Brazil	Ecological Time-Series Study	Climatic, environmental, social factors	Monthly	Statistical models (ARIMA, ETS, TBATS, BATS, STLM, StructTS, NNETAR, ELM, MLP, null model)	MAPE, Relative MAPE, Theil's U	Dengue cases	ARIMA and TBATS are the best models in various time horizons (12 months, 6 months, dan 3 months) Model accuracy not mentioned	Internal only
[35]	China	Time series analysis	Imported cases, Minimum temperature, Accumulative precipitation	Monthly	Time series Poisson regression	R^2	Dengue outbreaks	R^2 = 0.98 (98%)	Internal only
[36]	Taiwan	Observational Study	Meteorological variables, AQI, vector data	Daily	Machine Learning (Random Forest, XGBoost, Logistic Regression)	AUC	Dengue fever incidence	Random Forest: AUC = 0.9547, Accuracy = 89.94% XGBoost: AUC = 0.9329 Logistic Regression: AUC = 0.7905	Internal only
[37]	Taiwan	Observational Study	Minimum temperature, Maximum cumulative rainfall	Yearly	Poisson Regression	MSE	Dengue incidence	MSE for validation set = 2.21 MSE for training set = 2.11	Internal only
[38]	Vietnam	Observational Study	Climate variables (temperature, humidity, precipitation), time-shifted variables	Weekly	SARIMAX XGBoost LSTM Negative Binomial Regression	MAE, RMSE, AIC	Weekly dengue case counts	SARIMAX = 25.678 (83.33%) XGBoost = 21.409 (100%) LSTM = 30.456 (70.34%) Negative Binomial Regression = 22.345 (95.78%)	Internal only
[39]	Malaysia	Time Series Analysis	Epidemiological (notified cases, onset cases, interventions), Environmental (rainfall, temperature, humidity)	Weekly	Random Forest Support Vector Machine (SVM) Artificial Neural Network (ANN) Autoregressive Distributed Lag (ADL) Hierarchical Forecasting (Optimal Combination) Hierarchical Forecasting (Bottom Up)	MAPE	Dengue outbreak forecasting	Random Forest = 95% (with all factors) SVM = 92.47%; ANN = 86.10% ADL = 85.70% Hierarchical Forecasting (Optimal Combination) = 85.67% Hierarchical Forecasting (Bottom Up) = 84.85%	Internal only
[40]	Sri Lanka	Time Series Analysis	Historical dengue incidence data	Weekly	Modified ARIMA (Statistical)	MAPE	Dengue incidence forecast	MAPE: 1.554 (44.6%) (Validation), 0.3184 (Training) (68.16%)	Internal only
[41]	Philippines	Hybrid Model Development	Dengue incidence, climate data, past incidence	Weekly	ARIMA, NNAR, ANN, SVM, LSTM	RMSE, MAE, SMAPE	Dengue outbreaks	Hybrid ARIMA-NNAR: ~85%	Internal only

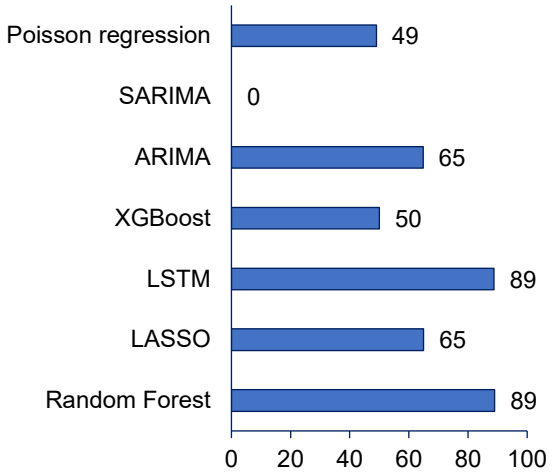

Fig. 4. Average model accuracy.

and 96.88%, respectively [28]. In Ba Ria Vung Tau Province, Vietnam reported temperature and humidity as reliable variables in predicting dengue cases, where the AUC and sensitivity were 90.00% and 85.00%, respectively [38]. Meanwhile, Taiwan showed that temperature and rainfall are important factors in predicting dengue cases, where the AUC and sensitivity are 88% and 80% respectively [37].

In general, the dengue case prediction models included in the studies demonstrated a relatively high level of predictive ability. However, the predictive accuracy of these models varies considerably depending on the specific model employed and the quality of the data used. The most commonly utilized statistical modeling techniques in dengue research are ARIMA, Generalized Additive Models (GAM), Negative Binomial Regression, and Poisson Regression. ARIMA and GAM are established models for examining the relationship between environmental factors and disease outcomes, as well as for conducting time series prediction analysis [44, 45]. According to recent literature, time series techniques are particularly considered effective in predicting the highly auto-correlated nature of dengue infections [46]. In recent years, data-driven techniques based on machine learning algorithms such as Random Forest, Decision Tree, Support Vector Machine (SVM), and Naïve Bayes have shown promising results in predictive analysis for classification problems [47].

More than half of the included studies rely on machine learning methods, particularly supervised learning models, to assess conventional and novel data streams. Supervised learning models are defined by the use of labeled data sets to train algorithms to accurately classify data or predict outcomes [21]. The advantages of machine learning techniques that demonstrate lower error rates in comparison to conventional statistical-based models in predicting dengue cases are manifold. In the era of big data, this technique can utilize the availability of data and, in addition to being non-parametric, it can also provide leeway in terms of strict assumptions [7]. Random forest, neural network, gradient boosting, and support vector algorithms are part of important machine learning algorithms, which have made significant contributions to several areas of public health, especially in forecasting infectious diseases such as COVID-19 [48], malaria [49], and have similar uses for making dengue outbreak predictions [7].

In some of the studies included in this literature, we assume that the machine learning method using random forest is the best method at the moment. Findings in Brazil state that the accuracy of this model in recognizing dengue cases is more than 90% [33]. Likewise, findings in Malaysia state that the accuracy of this model reaches 95% [39]. Similar findings in another study in Singapore, which stated that the potential of random forest and its strong predictive ability in clustering the

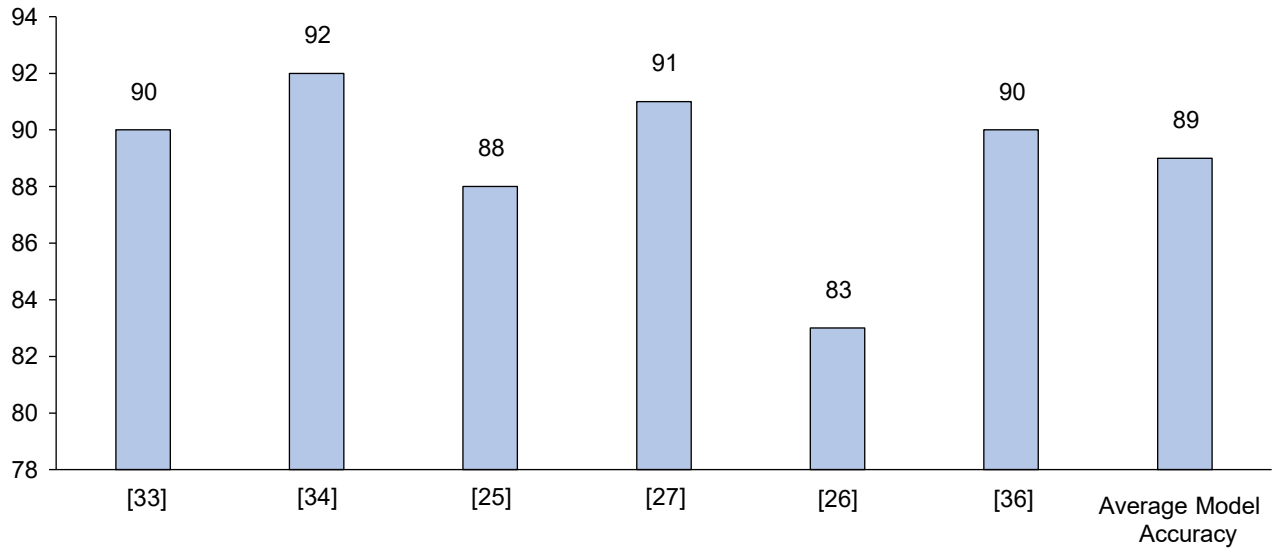

Fig. 5. Random forest model accuracy.

spatial risk of dengue transmission in Singapore. The dengue risk map generated using random forest has high accuracy and is a good tool to guide vector control operations, allowing targeted preventive measures before and during dengue outbreaks [50].

All studies employed internal validation to assess the accuracy of their findings. The utility of a forecasting model is contingent upon the certainty of its accuracy, or the extent to which it can predict real-world outcomes [51]. It is notable that the majority of published models have not undergone or been subjected to real-world validation. It is reasonable to conclude that models are unlikely to perform as well in real-world samples as they do in derived samples. This discrepancy, or validity shrinkage, is often significant. Consequently, it would be beneficial for future models to include mechanisms for estimating and reporting potential validity shrinkage, as well as predictive validity, in real-world data [52, 53]. External validation, on the other hand, was only used in a few studies that included [25–27]. This is despite the fact that external validation is considered very important for model development and is a key indicator of model performance by highlighting its applicability to participants, centers, regions or environments [54], It is imperative that external validation be employed during the process of model redevelopment. This entails making adjustments, updates, or recalibrations to the original model based on validation data, with the objective of enhancing its performance [55].

It should be noted that this systematic review is not without limitations. Firstly, the majority of the included studies originate from Asia, which encompasses a multitude of non-English speaking countries. Consequently, this review may have overlooked a substantial corpus of related literature published in other languages. Secondly, the inclusion criteria stipulated the necessity for studies to be derived from primary research in peer-reviewed journals. Consequently, preprints and grey literature, such as conference abstracts, committee and government reports, were excluded. It is therefore possible that some studies may have been omitted from our review.

Conclusion

The forecasting of dengue cases is a valuable resource for policymakers engaged in the formulation of strategies for the prevention of dengue outbreaks, particularly in regions where the disearse is endemic. The results of this systematic review indicate that the machine learning method utilizing the random forest algorithm is more effective than others method, particularly in comparison to statistical methods. Furthermore, this systematic review presents evidence of predictors in dengue case experience that focuses on incorporating climatic factors to create an early warning system, which can be utilized as a reference for preventing dengue transmission. The findings from this review have the potential to form the basis for more effective modelling practices in the future. These findings will contribute to the development of robust modelling across differenctt settings and populations and have significant implications for planning and decision-making processes for early dengue intervention and prevention.

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Whole-genome sequencing of two clinical strains of *Mycobacterium tuberculosis* with phenotypic susceptibility to rifampicin but predicted resistance by Xpert MTB/RIF

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Abstract

Introduction. More than 40% of *Mycobacterium tuberculosis* strains are resistant to rifampicin (RIF) and isoniazid, the first-line drugs. The tuberculosis pathogen becomes resistant to RIF mainly due to mutations in the *rpoB* gene. **The aim** of the study was to search for the most probable compensatory mutations in the *rpoA*, *rpoB* and *rpoC* genes encoding α-, β- and β′-subunits of *M. tuberculosis* RNA polymerase.

Materials and methods. A cross-sectional analysis of phenotypic and genetic resistance to RIF among 2298 clinical strains of *M. tuberculosis* revealed 8 cases in which resistance as determined by the Xpert Ultra MTB/RIF test was not confirmed bacteriologically. In all cases, these were chronic multidrug-resistant or extensively drug-resistant *M. tuberculosis* patients in whom RIF was discontinued due to the detection of resistance to this drug in the isolated strains. Two strains were obtained for genotype testing, Sanger sequencing and whole-genome sequencing.

Results. Repeat Xpert Ultra MTB/RIF test, Sanger sequencing and whole genome sequencing revealed the presence of a single *S450L* mutation in the *rpoB* gene with phenotypic sensitivity in both strains. Phylogenetic analysis revealed that both genomes belonged to the Beijing B0/W148 genotype. The strains were characterized by a higher growth rate than the other isolates. Two potential compensatory mutations *V483G* and *H748P* in the *groC* gene were identified in the absence of other significant changes in the *rpoA* and *rpoB* genes.

Conclusion. It is suggested that the phenomenon of discrepancy between results of bacteriological and molecular genetic tests is associated with the acquisition of compensatory mutations in the *groC* gene during RIF treatment of Beijing B0/W148 strains, and the identified mutations affect the conformation of the β'-subunit, restoring the transcription efficiency of affected by the major *S450L* mutation.

Keywords: *Mycobacterium tuberculosis, Beijing B0/W148, rpoA, rpoB, rpoC, compensatory fitness mutations*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (protocol No. 2, February 18, 2020).

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Оригинальное исследование
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Полногеномное секвенирование двух клинических штаммов *Mycobacterium tuberculosis* с фенотипической чувствительностью к рифампицину при прогнозируемой Xpert MTB/RIF устойчивости

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Аннотация

Введение. Более 40% штаммов *Mycobacterium tuberculosis* устойчивы к рифампицину (RIF) и изониазиду — препаратам первого ряда. Возбудитель туберкулёза приобретает устойчивость к RIF главным образом за счёт мутаций в гене *rpoB*.

Цель исследования — поиск наиболее вероятных компенсаторных мутаций в генах *rpoA*, *rpoB* и *rpoC*, кодирующих α-, β- и β′-субъединицы РНК-полимеразы *M. tuberculosis*.

Материалы и методы. Перекрёстный анализ фенотипической и генетической устойчивости к RIF среди 2298 клинических штаммов *M. tuberculosis* выявил 8 случаев, когда устойчивость, определённая тестом Xpert Ultra MTB/RIF, не подтверждалась бактериологическим методом. Во всех случаях это были хронические больные туберкулёзом с множественной или широкой лекарственной устойчивостью, у которых был отменён RIF по причине обнаружения устойчивости к этому препарату у выделенного штамма. Для исследования генотипа, секвенирования по Сэнгеру и полногеномного секвенирования были получены 2 штамма.

Результаты. Повторный тест Xpert Ultra MTB/RIF, секвенирование по Сэнгеру и полногеномное секвенирование выявили наличие единственной мутации *S450L* в гене *rpoB* при наличии фенотипической чувствительности у обоих штаммов. При филогенетическом анализе выяснено, что оба генома принадлежали к генотипу Beijing B0/W148. Штаммы отличались более высокой скоростью роста, чем другие изоляты. Выявлены две потенциальные компенсаторные мутации *V483G* и *H748P* в гене *rpoC* при отсутствии других значимых изменений в генах *rpoA* и *rpoB*.

Заключение. Высказано предположение, что феномен расхождения бактериологических и молекулярно-генетических результатов связан с приобретением в процессе лечения RIF штаммами Beijing B0/W148 компенсаторных мутаций в гене *rpoC,* а выявленные мутации влияют на конформацию β'-субъединицы, восстанавливая эффективность транскрипции, вызванную мажорной мутацией *S450L*.

Ключевые слова: *Mycobacterium tuberculosis, Beijing B0/W148, rpoA, rpoB, rpoC, компенсаторные фитнес-мутации*

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Introduction

Multidrug-resistant (MDR) tuberculosis develops in patients treated with rifampicin (RIF) and isoniazid, the most effective anti-tuberculosis drugs, also called first-line drugs. Globally, more than 40% of *Mycobacterium tuberculosis* (MBT) strains become MDR or at least RIF resistant[1]. RIF binds close to the active site in the β subunit (*rpoB* gene) of the bacterial RNA polymerase enzyme [1] in the RIF resistance determining region (RRDR). The binding of RIF to RRDR sterically impedes the elongation of newly synthesized RNA, which ultimately blocks protein synthesis by the microbial cell. MBT has no known mechanism of horizontal gene transfer; RIF resistance mainly arises from chromosomal mutations within the RRDR [2]. The impact of RIF resistance is significant and is often reflected in MBT in terms of a reduced growth rate and the decreased competitiveness of RIF-resistant mutants compared to ancestral susceptible forms [3]. However, it has been observed that MBT forms with low adaptability can partially or completely restore phenotypic properties over time, in particular, increase growth rate due to the appearance of so-called compensatory mutations [4]. The identification of compensatory mutations is very difficult and depends on the methodology used.

Molecular epidemiologic studies of Beijing B0/W148 genomes belonging to the L2 genetic lineage [5, 6] indicate that more than 95% of clinical strains of this genotype contain mutations in RRDR and is one of the key factors in the epidemic spread of primary drug-resistant tuberculosis with RIF in Russia [6, 7]. Almost 90% of these strains carry the most common amino acid substitution *S450L* (nucleotide substitution C→T in 761155 position of the genome) [6], and it was noted that the genetic cost of this substitution in the RRDR of the *rpoB* gene for mutants is the lowest [2].

The aim of the study: to search for the most probable compensatory mutations in *rpoA*, *rpoB* and *rpoC* genes encoding α-, β- and β′-subunits of RNA polymerase of MBT, causing the phenomenon of phenotypic sensitivity to RIF.

Materials and methods

A retrospective cross-analysis of Xpert Ultra MTB/RIF and phenotypic bacteriological results for 2022 obtained in the laboratory department of the Irkutsk Regional Tuberculosis Hospital was performed. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (protocol No. 2, February 18, 2020).

2298 samples were examined, of which 529 were sensitive to RIF, while 363 were resistant; sensitivity to RIF using Xpert Ultra MTB/RIF was not determined in 90 samples. The main reason for the lack of positive PCR results is the low concentration of the target substance when Xpert Ultra MTB/RIF was performed. In 8 cases, resistance determined by Xpert Ultra test in sputum was not confirmed by bacteriologic methods. All cases were chronic MDR or extensively drug-resistant TB patients who had been discontinued from RIF due to the presence or acquisition of resistance to the drug in a previously isolated strain.

Two strains were obtained for repeat Xpert Ultra MTB/RIF, genotype testing, Sanger sequencing, and whole genome sequencing (WGS) (**Table 1**). DNA isolation, library preparation, WGS and bioinformatics, phylogenetic and statistical analyses were performed as described previously [6]. Primary nucleotide sequences were deposited in the NCBI bioproject PRJNA1215569. Resistance to anti-tuberculosis drugs was determined on a BD Bactec bacteriological analyzer (Becton Dickinson) and on Löwenstein–Jensen medium according to the Order of the Ministry of Health of Russia from 21.03.2003 No. 109 (ed. 05.06.2017). Genetic heteroresistance in individual genome positions was determined by the number of alternative short reads during WGS as described previously [8].

Amino acid substitution probability in detected mutations was investigated using two approaches: PAM matrices (Point Accepted Mutation matrices) — PAM30 and PAM250 [9] and SIFT (Sorting Intolerant From Tolerant) algorithm for predicting amino acid substitutions affecting protein function [10].

Results

Repeated Xpert Ultra MTB/RIF test, Sanger sequencing and WGS revealed the presence of a single *S450L* mutation in the *rpoB* gene with phenotypic sensitivity in both strains. Phylogenetic analysis revealed that both genomes belonged to the Beijing B0/W148 genotype. The strains were characterized by a higher growth rate than the other isolates. After elucidation of the genotypic affiliation of the studied strains to the Beijing B0/W148 genotype, 513 complete B0/W148 genomes from the Short Read Archive (NCBI) online service published between 1995 and 2020 for strains from Northern Eurasia were used as reference genomes.

A total of 34 missense mutations in the *rpoB* gene were detected for this genome set [11]. Level 1 missense mutations were in 9 variants; level 2 — in 2; and level 3 — in 20 (**Table 2**). Furthermore, 3 mutations were found in the *rpoB* gene that were absent in the WHO catalog description: *E82G, I90M, R219G*. 45 missense mutations were detected in the *rpoC* gene, all of them belonged to mutations of level 2 significance (Table 1). Only 5 missense mutations were detected in

[1] WHO. Global tuberculosis report 2024. Geneva: World Health Organization; 2024. URL: https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2024

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Table 1. Characterization of *M. tuberculosis* isolates

No.	Patient record group	HIV	Drug resistance	Genotype
Irk1	Ineffective course of tuberculosis treatment	+	To isoniazid, RIF*, capreomycin, pyrazinamide, prothianamide, bedaquiline, linezolid	Beijing B0/W148
Irk2	Tuberculosis relapse	_	To isoniazid, RIF*, ethambutol, capreomycin, pyrazinamide, levofloxacin, bedaquiline, linezolid	Beijing B0/W148

Note. *Based on the Xpert Ultra MTB/RIF results, but not the microbiological test.

Table 2. Presence of mutations of the 1st, 2nd and 3rd levels of significance [11]

Gene	Mutations of level 1 significance	Mutations of level 2 significance	Mutation of level 3 significance
rpoB	*L430P; Q432P; D435V; D435Y; H445D; H445L; S450L; L452P; H723D*	T427A; S431R	*P45S; G79S; V305I; G376V; T400A; P454S; I491M; V496A; L554P; Y564H; S672Y; L731P; V800A; R827C; R827L; H835P; G836S; K891E; Q980K; R1008C*
rpoC	None	*E187G; G311R; G332S; G433C; P434A; P434L; K445R; L449R; F452C; V483G; D485N; E488Q; I491V; I491T; L507V; L516P; V517L; G519S; A521D; Q523E; H525N; L527V; L558L; Y586H; Q693H; N698H; N698S; N698K; E702K; D735N; D735E; D747A; H748P; E757A; R770H; T812I; S838C; D943N; D943G; M983I; P1040S; P1040R; I1046M; V1147A; K1152N*	None
rpoA	None	*G31C; R153R; T187P; V183A; R182Q*	None

the *rpoA* gene, also belonging to mutations of the 2nd level of significance (Table 1).

The following combinations of mutations were detected in the 2 strains studied: in the Irk1 strain, *rpoB — S450L*; *rpoC — H748P*; in the Irk2 strain, *rpoB — S450L*; *rpoC — V483G*. Interestingly, a similar case of drug sensitivity in the presence of a combination of *rpoB — S450L*; *rpoC — V483G* mutations was described in 2024 in the strain of Euro-American lineage (4.2.2.2.2.1) [12]. However, the authors suggested that the result obtained was a laboratory error due to the use of inflated concentrations of RIF during testing. The *H748P* mutation in the *rpoC* gene is not considered compensatory in the final version of the article, although it was described as such in the original manuscript by the same authors[2] [12].

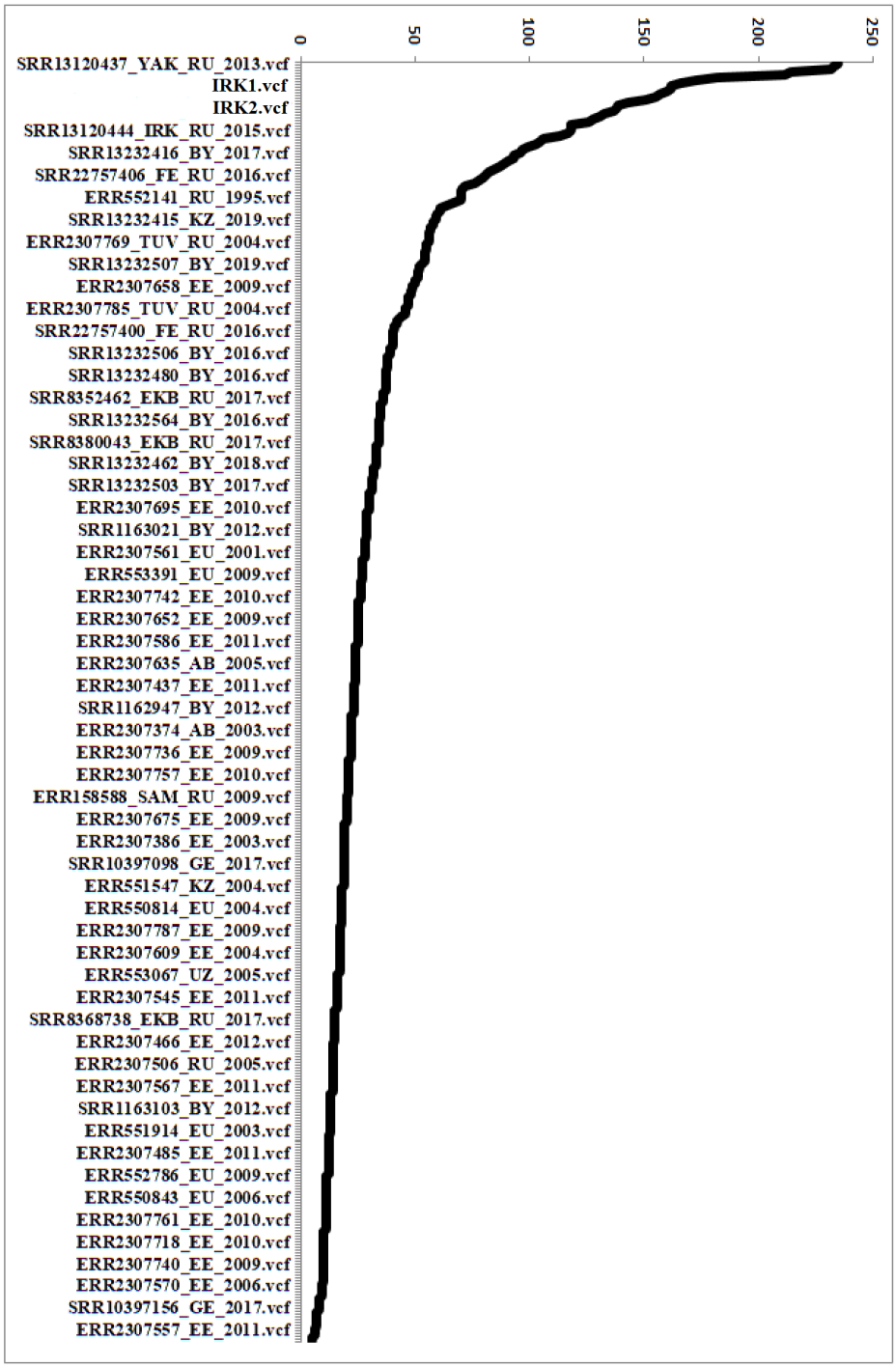
The Irk1 and Irk2 genomes tested occupy the highest positions in terms of heteroresistance values (214 and 212) at position 761155 (*rpoB* nucleotide substitution — *S450L*; **Figure**). The highest heteroresistance (235), expressed as the presence of alternative short reads at WGS at the position under study, was observed in only one genome from Yakutia isolated in 2013. We investigated the probability of occurrence of detected amino acid substitutions in the *rpoC* gene using two approaches: PAM (Point Accepted Mutation) matrices — PAM30 and PAM250 [9] and the SIFT (Sorting Intolerant From Tolerant) algorithm [10]. To predict amino acid substitutions affecting protein function, PAM matrices were used to estimate the probability of amino acid substitutions during evolution [9]. The SIFT algorithm was used to determine whether amino acid substitutions affect protein function using evolutionary information and homologous sequence alignments [10].

A SIFT value of 0.00 was obtained for *V483G* (IRK2) indicating low tolerance, which may indicate a significant effect of this substitution on the function of the β'-subunit of RNA polymerase. However, the moderate values of PAM10 (0) and PAM250 (–1) suggest that this mutation does not lead to a complete loss of function and may stabilize the RNA polymerase complex, compensating for the destabilization caused by *S450L*. In turn, the *H748P* (IRK1) mutation with SIFT 0.05 and PAM250 (-3) shows moderate tolerance, indicating a slightly negative effect on the protein. It can be hypothesized that the above mutations affect the conformation of the β'-subunit, restoring the transcription efficiency caused by the major *S450L* mutation, where *V483G* may play a more pronounced compensatory role.

Discussion

The phenomenon of the emerging sensitivity in strains has been observed earlier in two international projects [8] when the minimum inhibitory concentration of MBT strains from the same patient was determined sequentially. It has been repeatedly observed (data not published) that withdrawal of certain anti-tuberculosis drugs, including RIF, leads to a decrease in the minimum inhibitory concentration down to the values of the borderline sensitivity defined by the manufacturer of the Sensititre MYCOTB kits (TREK Diagnostics). The main hypothesis that could explain this phenome-

[2] URL: https://www.biorxiv.org/content/10.1101/2022.02.22.481565v1.full.pdf



Heteroresistance assessment of a sample of 515 genomes at position 761155 (*rpoB* nucleotide substitution — *S450L*).

non was the assumption that antibiotic-sensitive clones begin to multiply more actively in the pathogen population after anti-tuberculosis drug withdrawal from the persister pool [8]. The Beijing B0/W148 genotype carries resistance to RIF in more than 95% of cases upon primary infection, i.e. it has already acquired all the compensatory mutations necessary for survival outside the organism in the process of evolution. The key question of the study is what fitness mutations lead to the emergence of the sensitivity phenomenon in the presence of the major *rpoB* mutation — *S450L*. The detected missense mutations *V483G* and *H748P* in the *groC* gene in the absence of other significant changes in the *rpoA* and *rpoB* genes may indicate that the withdrawal of certain anti-tuberculosis drugs may lead to the emergence of compensatory fitness mutations, manifested as sensitivity to anti-tuberculosis drugs in the presence of the major substitution, identified in PCR test. It can also be assumed that against the background of high heterogeneity on the *rpoB* gene in the pathogen population,

the described combination of *S450L* mutations together with *V483G/H748P* undergoes stabilizing selection only during RIF treatment. The abolition of RIF leads to a gradual return of the pathogen population to a more stable model in which clones containing *V483G/H748P* and other fitness mutations are eliminated. The source of this is persisters harboring *S450L* but lacking mutations in the *rpoC* gene.

Conclusion

It can be assumed that the phenomenon of discrepancy between results of bacteriological and molecular genetic tests is associated with the acquisition of compensatory mutations in the *groC* gene during RIF treatment of Beijing B0/W148 strains. The identified mutations affect the conformation of the β'-subunit, restoring the transcription efficiency caused by the major *S450L* mutation. Further studies of the phenomenon of decreased resistance to anti-tuberculosis drugs in the tuberculosis pathogen after its withdrawal are necessary.

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Investigation of the pathogenic potential and the possibility of cross-species transmission of H5 avian influenza viruses detected on the territory of the Russian Federation in 2018–2022

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Abstract

Introduction. The rapid evolution of highly pathogenic avian influenza (HPAI) viruses through antigenic drift and reassortment can lead to enhanced replication efficiency and cross-species transmission to mammals, as evidenced by recent outbreaks in various animal populations. Identifying mammalian pathogenicity markers in circulating HPAI viruses is crucial for evaluating their pathogenic potential and ability to cross species barriers.

The aim. This study analyzed genomic sequences of highly pathogenic H5 avian influenza virus (AIV) isolates collected in the Russian Federation between 2018 and 2022.

Materials and methods. We utilized original complete genome sequencing data alongside with nucleotide sequences of H5 AIV isolates and strains available in public databases.

Results. Analysis revealed a predominance of viruses with replication complexes adapted to avian cells. Examination of viral hemagglutinin amino acid sequences showed that most strains maintained receptor-binding sites of avian origin, with enhanced affinity for SAα-2,3-Gal receptors present in avian epithelial cells. However, we identified several mammalian virulence factors that have emerged and spread within the avian influenza virus population, including full-length active PB1-F2 protein, a 5-amino-acid insertion in the NS1 protein, and specific amino acid substitutions in the M1 protein.

Conclusion. The presence of mammalian pathogenicity factors in the avian influenza virus population may facilitate successful cross-species transmission through suppression of specific immune responses, followed by adaptation of viral hemagglutinin to mammalian cell receptors through antigenic drift and natural selection. The observed elimination of certain adaptive mutations from the avian influenza virus population validates the effectiveness of stamping-out policies and vaccination restrictions in industrial poultry farming as important measures to mitigate the zoonotic potential of avian influenza.

Keywords: *avian influenza, genetic analysis, amino acid substitutions, adaptive mutations, cross-species transmission of the virus*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the Federal Centre for Animal Health (protocol No. 17, April 24, 2023).

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Оригинальное исследование
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Изучение патогенного потенциала и возможности межвидового перехода вирусов гриппа птиц подтипа H5, выявленных на территории России в 2018–2022 годах

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Аннотация

Введение. Высокая скорость эволюции вирусов высокопатогенного гриппа птиц (ВПГП), обусловленная антигенным дрейфом и реассортацией, может привести к устойчивой репликации и передаче вируса млекопитающим, что наблюдается в популяциях животных в последние годы. Исследование маркеров патогенности для млекопитающих у циркулирующих вирусов ВПГП даёт возможность оценить их патогенный потенциал и способность к межвидовому переходу.

Цель работы — провести анализ геномных последовательностей изолятов вируса гриппа птиц (ВГП) подтипа H5, выявленных на территории России в 2018–2022 гг.

Материалы и методы. В работе использованы результаты собственного полногеномного секвенирования и нуклеотидные последовательности изолятов и штаммов ВГП подтипа H5, опубликованные в открытых базах данных.

Результаты. Установлено, что преобладают вирусы с репликативным комплексом, адаптированным к размножению в клетках птиц. Анализ аминокислотной последовательности вирусного гемагглютинина выявил доминирование в рецептор-связывающем сайте белка аминокислот, характерных для ВГП и обеспечивающих повышенное сродство к рецепторам SAα-2,3-Gal эпителиальных клеток птиц. Показано появление и распространение в популяции ВГП факторов вирулентности для млекопитающих, таких как полноразмерный активный белок PB1-F2, дополнительная вставка из 5 аминокислот в белке NS1 и аминокислотные замены в белке M1.

Заключение. Наличие в популяции ВГП факторов патогенности для млекопитающих может способствовать успешному межвидовому переходу вируса за счёт подавления отдельных элементов иммунной защиты с последующей адаптацией вирусного гемагглютинина к клеточным рецепторам млекопитающих в результате антигенного дрейфа с дальнейшим закреплением приобретённых мутаций естественным отбором. Элиминация из популяции ВГП ряда адаптационных мутаций, способствующих размножению ВГП в клетках млекопитающих, подтверждает эффективность стратегии стемпинг аут и запрета на вакцинацию в промышленном птицеводстве в качестве сдерживающего фактора для гриппа птиц как зооантропонозного заболевания.

Ключевые слова: *грипп птиц, генетический анализ, аминокислотные замены, адаптационные мутации, межвидовой переход вируса*

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Introduction

Avian Influenza Virus (AIV) is the pathogen of a dangerous highly contagious disease of domestic and wild birds, characterized mainly by respiratory and digestive tract damage. In case of infection with highly pathogenic avian influenza (HPAI) viruses of H5 or H7 subtypes, bird mortality reaches 100%. In 1996, an influenza virus of the H5N1 A/goose/Guangdong/1/1996 subtype was discovered in China, which was subsequently recognized as the founder of the HPAI genetic lineage Gs/Gd/96. Over time, virus isolates of this genetic lineage became widespread not only in Asian countries but also worldwide. Thus, from 2005 to 2007, outbreaks of disease caused by this virus subtype caused significant damage to the poultry industry in Russia. Since 2014, the H5N8 subtype has been detected in Russia, whereas the H5N5 subtype has been detected there since 2016 and the H5N6 subtype — since 2018. In 2018–2019, outbreaks of HPAI (subtypes H5N1, H5N6 and H5N8) were registered among wild and domestic poultry in Asian and African countries, on the territory of Russia – in the Central, Southern, Volga and Far Eastern Federal Districts, including in farm birds at poultry farms (subtype H5N8). In 2020, H5N8 subtype HPAI was spread widely in the countries of Europe and the Middle East, on the territory of Russia and Kazakhstan [1, 2]. Furthermore, in Omsk, Rostov and Astrakhan regions, H5N5 subtype was detected. At the end of 2020, the H5N8 virus was detected in people who came into contact with sick poultry at a poultry farm in the Astrakhan region [3, 4].

In 2021–2022, H5N1 subtype influenza became widespread, with outbreaks reported in Europe, Asia, Africa, and North America [4]. Influenza outbreaks among mammals such as mink, foxes, and fur seals were of great concern [5-9]. The isolated virus has been found to have substitutions that indicate adaptation to reproduction in mammals. At the moment, the H5N1 subtype is represented in the territory of Russia. In August 2023, a dead fur seal was found on the territory of Sakhalin Island[1], and examination of pathological material from the animal showed the presence of avian influenza virus of the H5N1 subtype.

In the spring of 2024, for the first time in the United States, HPAI H5N1 virus was detected in cows on a dairy farm. Clinical signs included mastitis, lethargy, decreased feed intake, diarrhea and nasal discharge. Since then, the virus has been detected on dairy farms in at least 13 U.S. states, and environmental release of the virus with milk has also been confirmed. The identified virus has been assigned to genetic clade 2.3.4.4b and genotype B3.13, circulating in wild and domestic birds in North American countries since 2021 [10, 11]. After a certain amount of time, the HPAI H5N1 virus was detected in sick and dead cats [12] and a human dairy farm worker. The virus identified in human samples had an amino acid substitution in the PB2 protein (E627K), which is associated with viral adaptation to mammalian hosts and has previously been found in humans and other mammals infected with H5N1 and other subtypes of H5N1 and other subtypes of type A viruses, including H7N9 and H9N2 [13]. The transmission of HPAI from birds to mammals and then proven cross-species transmission from cows to cats and humans indicates a significant threat to public health.

The presence in influenza type A virus of a polybasic proteolytic cleavage site of the hemagglutinin protein provides the possibility of an extensive infectious process affecting various organs and tissues in different animal species. The ability of the virus to replicate and counteract the host immune response is provided by other viral proteins. Previously, various groups of scientists have identified specific amino acid substitutions that enable replication in mammals, counteraction to the immune response, and a more severe course of the infectious process [14–54].

The aim of this study was to analyze the genome of HPAI detected in Russia for the presence of pathogenicity markers potentially contributing to overcoming the cross-species barrier from birds to mammals and to assess the pathogenic potential of circulating viruses as agents of zooanthroponotic disease.

Materials and methods

Biomaterial

In this study, isolates of H5 subtype HPAI isolated from biomaterial from birds at ARRIAH in 2018–2022 were investigated. Virus-containing allantois fluid of pathogen-free chicken embryos or, if it was impossible to isolate viruses on chicken embryos, pathologic material from birds (cloacal and tracheal flushes, 10–20% organ suspensions prepared on the basis of 0.9% solution of NaCl) were used as material. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the Federal Centre for Animal Health (protocol No. 17, April 24, 2023).

RNA isolation

Total RNA was isolated using RIBO-prep reagent kit for RNA/DNA isolation from clinical material (Central Research Institute of Epidemiology of Rospotrebnadzor).

Reverse transcription and polymerase chain reaction

Real-time polymerase chain reaction with reverse transcription (RT-PCR-RT) were performed in one stage

[1] World Organisation for Animal Health. Event 5191. https://wahis.woah.org/#/in-event/5191/dashboard

using amplification reagents (Syntol) and primers and probes for amplification of *MP* and *HA* gene fragments.

The RT reaction was performed in two steps (primer annealing and RT itself) using the Maxima H Minus Reverse Transcriptase reagent kit (includes RT buffer and Maxima H revertase; Thermo Fisher Scientific), RiboLock RNase Inhibitor (Thermo Fisher Scientific), dNTPs solution (Syntol), RNase-free bidistilled water, and a solution of direct specific segment-universal primers for amplification of all segments of type A HPAI. Classical PCR was performed using amplification reagents (Syntol) and specific segment-universal primers for amplification of all segments of type A HPAI. PCR products were purified from the PCR mixture using the Wizard(R) SV Gel and PCR Clean-Up System kit (Promega).

Sequencing

Whole-genome sequencing was performed using a MiSeq genetic analyzer (Illumina) according to the instructions for the instrument. Nextera XT and Nextera XT Index Kit commercial kits (Illumina) were used for library preparation.

Nucleotide sequences

The results of in-house whole genome sequencing and nucleotide sequences of isolates and strains of H5 subtype HPAI from Russia, published in the GenBank database of the NCBI electronic resource[2] and EpiFlu platform[3] (see Appendix on the journal's website https://microbiol.crie.ru/jour) were used in this study.

The nucleotide and corresponding amino acid sequences were analyzed using the BioEdit v. 7.0.5.3 program. Sequences were aligned using the ClustalW multiple alignment program. The phylogenetic tree was constructed using the NJ algorithm in the implementation of the MEGA v. 7.06 package.

Results

As a result of studies conducted in 2018-2022 and covering all federal districts of the Russian Federation, ARRIAH specialists identified 1,082 samples that contained genetic material of H5 subtype HPAI (**Table 1**).

H5 subtype HPAI were detected predominantly in samples from poultry throughout the study period (Table 1). Some viruses (45) were subjected to whole-genome sequencing to study virus evolution and characterize their biological properties; the sample was compiled on the basis of geographical distribution and differences in virus subtypes by neuraminidase. To expand the study sample, whole-genome sequences of H5 subtype HPAI detected in Russia from 2018 to 2022 (134 isolates) available in public databases were retrieved. It is necessary to emphasize that the use of the terms "population", "virus circulation" is not correct for the avian influenza virus, which is capable of spreading over vast territories during one season of wild bird migration. In this case, the term "avian influenza virus population" will be understood as a set of 190 viruses detected in Russia in 2018–2022, for which whole-genome sequences were obtained. The term "HPAI population" does not imply the presence of foci of persistent illness and long-term circulation of avian influenza viruses on the territory of Russia.

Based on the analysis of the predicted amino acid sequence, the cleavage site of the viral hemagglutinin of the compared isolates was determined. For all viruses it had a similar structure containing 6 basic amino acids with a variation at position 342 — RE(K/R) RRKR. The exception was A/dalmatian pelican/Astrakhan/417-1/2021 (H5N5) virus, whose cleavage site contained 7 basic amino acids RKKRRKR. The amino acid motif $G_{225}QRG_{228}$ (according to H3 subtype numbering) was detected in the receptor-binding part of the viral protein in all viruses.

H5 subtype HPAI are capable of infecting mammals, including humans, despite the fact that their hemagglutinin predominantly interacts with cellular SAα-2,3-Gal receptors. However, in the case of successful reproduction of HPAI in mammalian cells, researchers have identified mutations in other viral genes that are considered markers of HPAI adaptation for reproduction in mammals. According to their phenotypic manifestation, the marker substitutions can be divided into two main groups: mutations associated with increased activity of the viral polymerase complex in mammalian cell culture (CC), and mutations that enhance the virulent properties of the virus during experimental infection of laboratory mice and cause changes in metabolism at the organismal level associated with modification of the immune response in the host organism. Amino acid substitutions for which a change in the biological properties of the virus has been experimentally demonstrated and a link between the mutation and its phenotypic manifestation was established were included in the analysis. **Table 2** shows the positions of amino

Table 1. Results of samples testing for the presence of the H5 subtype HPAI genome in Russia in 2018–2022

Year	Number of samples analyzed	Number of samples containing HPAI/H5	
		from domestic birds	from wild birds
2018	2749	208	0
2019	5558	2	0
2020	6288	222	27
2021	6418	297	56
2022	6087	250	20
Total	27,100	979	103

[2] URL: https://www.ncbi.nlm.nih.gov/nucleotide/
[3] URL: https://www.gisaid.org/

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Table 2. Marker amino acid substitutions associated with HPAI adaptation to reproduction in mammalian CC

Protein	Amino acid's position number and HPAI isolates containing mutations	Phenotypic manifestation of the mutation
PB1	*622G* — all studied viruses	Increased polymerase activity [39]
	678S — all studied viruses, except *678N* — A/turkey/Rostov-on-Don/332-XX/2021, *678G* — A/dabchick/Tyva/767-58/2021	*678N* — increased polymerase activity [19]
PB2	*89V*, *309D, 339K*, *477G* — all studied viruses, *495V/I/A, 676T/I/M/A*	Aggregate mutations: *89V, 309D, 339K, 477G, 495V, 676T* — increased polymerase activity and replication in mammalian CC [15]
	292I/T, *588A* — all studied viruses, except *292V* — H5N8 2018–2020 (except chickens from Novosibirsk in 2020), A/chicken/Kostroma/1761-1 (H5N8), A/chicken/Tomsk/1797-7/20 (H5N8), A/duck/KChR/1590-14/20 (H5N8), A/crow/Khabarovsk/2712-1/2022 (H5N1), A/dabchick/Tyva/767-58/2021 (H5), *588V,* A/common gull/Saratov/1676/2018 (H5N6)	*292V, 588V* — increased polymerase activity and replication in mammalian CC, increased virulence in mice [40]
	389R, 598T — all studied viruses	*389R, 598T* — increased polymerase activity and replication in mammalian CC at low temperatures [41]
	482K — all studied viruses, except 482R, A/chicken/Kostroma/304-XX/2020 (H5N8), A/chicken/Kostroma/ 1761-1 (H5N8), A/crow/Khabarovsk/776-56/22 (H5N1), A/duck/Magadan/2272-8/2022 (H5N1), A/goose/Magadan/2272-5/22 (H5N1), A/poultry/Magadan/1560-1/2022 (H5N1)	*482R* — increased polymerase activity in mammalian CC [42]
PA	*37A, 100V* — all studied viruses, except *37S,* A/turkey/Stavropol/165-5/2022	*37A, 100V* — increased polymerase activity and replication in mammalian CC, increased virulence in mice [18]
	97T — all studied viruses, except *97I,* A/Chicken/Ryazan/1093-1/2022 (H5N1), A/Poultry/Samara/1659-1/2022 (H5N1), A/Poultry/Samara/1643-1/2022 (H5N1), A/Chicken/Kursk/1281-1/2022 (H5N1), A/Goose/Saratov/1965-1/2022 (H5N1), A/Goose/Belgorod/1498-1/2022 (H5N1), A/Duck/Ivanovo/1462-3/2022 (H5N1), A/Duck/Belgorod/1482-10/2022 (H5N1), A/Chicken/Orel/1484-5/2022 (H5N1), A/Chicken/Kaluga/1424-2/2022 (H5N1), A/Chicken/Rostov/1724-2/2022 (H5N1)	*97I* — increased polymerase activity and replication in mammalian CC, increased virulence in mice [43]
	127V, 44V, *241C*, *343A, 573I* — all studied viruses, except: *127A* — all H5N8/2018 viruses *343A, 347D* — all studied viruses, except: *343T* — A/Crow/Khabarovsk/2712-1/2022 (H5N1), *343S* — H5N5 and H5N8 viruses, circulating in 2020–2021	*127A, 44I, 241Y, 343T, 573V* — increased replication in mammalian ECs, increased virulence in mice [16] *343S, 347E* — increased replication in mammalian CC, increased virulence in mice [44]
	142K, 147I, *171I*, *182M* — all studied viruses; *142R,* A/common gull/Saratov/1676/2018 (H5N6), *182L,* A/waterfowl/Russia/1526-4/2021 (H5N5), A/shelduck/Kalmykia/1814-1/2021 (H5N5)	*142R, 147V, 171V, 182L* — increased polymerase activity and replication in mammalian CC [45]
	224S/A — all studied viruses, *383D* — all studied viruses	*224P, 383D* — increased polymerase activity and replication in mammalian CC [17]
NP	*41I* — all studied viruses, except: *41V* — A/common teal/Chelyabinsk/1379-1/2021 (H5N1)	*41V* — increased polymerase activity in mammalian CC at low temperature [46]
NS1	*3P/S, 41K, 74D* — all studied viruses, except: *41R* — A/chicken/Tomsk/1797-7/20 (H5N8)	*3S, 41K, 74N* — enhanced replication in mammalian CC and pathogenicity to mice [47]
	55E, 66E, 138F — all studied viruses, except: *66K* — viruses H5N8 2020–2021 *138L* A/goose/Omsk/3003/2020 (H5N8) A/goose/Omsk/3008/2020 (H5N8)	*55E, 66E, 138F* — enhanced replication in mammalian CC, decreased response to interferon [48]

acid substitutions of HPAI proteins that promote HPAI reproduction in mammals.

Analysis of the predicted amino acid sequences of the polymerase complex proteins revealed single marker substitutions capable of enhancing the work of the virus replicative complex in mammalian cells, which were fixed in the avian influenza virus population. For example, only 1 substitution at position *622G* became fixed in the PB1 protein. Two other mutations (*678N* and *105S*) had a sporadic distribution.

Mutations *389R* and *598T* were fixed in the PB2 protein by natural selection. These mutations among avian influenza viruses have been recorded previously, but have now become dominant. The widespread distribution in 2018-2020 of the *292V* mutation and the appearance of single *482R* mutations in the population were noted. Analysis of the set of "adaptation mutations" *89V*, *309D*, *339K*, *477G*, *495V*, *676T* indicates the consolidation by natural selection of this set of amino acid substitutions. Experimental studies have shown that the set of these substitutions can compensate for the absence of lysine at position 627 of the PB2 protein for successful replication of HPAI in mammalian cells [15].

Analysis of the predicted amino acid sequence of the *PA* gene showed that the *383D* mutation was fixed in the population. A wide distribution of mutations *37A*, *61I*, *63V*, *100V*, *343S*, *383D* and single cases of mutations *224P*, *343T*, *142R* were detected. Although the largest number of "adaptation mutations" was found in the nucleotide sequences of the *PA* gene, this does not appear to be critical because they are randomly distributed among the viruses. Furthermore, a number of studies have shown the necessity of a synergistic effect for the phenotypic manifestation in mammals of "adaptation mutations" in the *PA* gene [16–18].

Analysis of marker amino acid substitutions associated with the virulent properties of HPAI showed that the 42S mutation in the NS1 protein is fixed in the HPAI population (**Table 3**). This substitution is a marker of virulent properties for mice and can counteract the induction of interferon in the host cell, as well as prevent activation of the NF-κB pathway during the immune response [19]. In addition, the amino acid substitutions *30D* and *215A* in the M1 protein, recognized as determinants of pathogenicity for mice, were detected in all isolates [20].

During the study of pathogenicity factors, the *NS1* gene encoding the corresponding protein with an additional 5 amino acid insertions at positions 80–84 was found to be fixed in the HPAI population. Experimental studies have shown that hybrid viruses with this insertion can induce a hyperimmune response in the organism, which is known as the cytokine storm [21]. The analysis showed that among influenza viruses, this mutation began to take hold after 2017.

Discussion

As a result of this study, it has been established that evolutionary selection has fixed a number of amino acid substitutions that contribute to the successful reproduction of HPAI in mammals. At the same time, questions remain about the mechanism of functioning of the viral receptor that allows the virus to make cross-species transmission. According to previous studies, the amino acid motif $G_{225}QRG_{228}$ is characteristic of influenza viruses isolated from birds and has high affinity for SAα-2,3-Gal group receptors [14, 22]. However, interpretation of the affinity properties of viral hemagglutinin to SAα-2,3-Gal group receptors or to SAα-2,6-Gal group receptors based on the primary amino acid sequence is difficult. It has been found that changes in the tropic

Table 3. Marker amino acid substitutions associated with increased HPAI virulence

Protein	Amino acid's position number and HPAI isolates containing mutations	Phenotypic manifestation of mutations
PB1	*105S* — A/chicken/Penza/300/2018	Increased virulence in mice [43]
PB1-F2	*66N* — all studied viruses, except: *66S* — HPAI isolates 2019-2022 subtypes H5N5, H5N1	*66S* — virulence and enhanced immune response in mice [34, 54]
NP	*319N* — all studied viruses, except: *319K* A/Crow/Khabarovsk/776-56/22 (H5N1) A/Duck/Magadan/2272-8/2022 (H5N1) A/Goose/Magadan/2272-5/22 (H5N1) A/Poultry/Magadan/1560-1/2022 (H5N1) A/Chicken/Ryazan/1093-1/2022 (H5N1)	*319K* — disruption of intranuclear transport in mammalian cells [23]
M1	*30D, 215A* — all studied viruses *43M* — all studied viruses	*30D, 215A* — increased virulence in mice Increased virulence in mice [49]
NS1	*42S* — all studied viruses	Increased virulence and decreased antiviral response in mice [50]
	92D — all studied viruses, except: 92E, A/common gull/Saratov/1676/2018 (H5N6)	*92D* — increased virulence in pigs and mice [51]
	103F/Y, 106M — all studied viruses	*103F, 106M* — increased virulence in mice [52, 53]

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properties of influenza viruses are possible as a result of both single mutations and a whole set of mutations in the amino acid sequence. In our unpublished studies and literature data comparing the predicted amino acid sequence of the *HA* gene of viruses isolated from birds and mammals, no amino acid substitutions were identified that were unique to AIV or unique to viruses isolated from mammals. Apparently, the hemagglutinin of influenza virus of genetic clade 2.3.4.4 as a result of single mutations is able to change its affinity properties with respect to sialic acid residues and, while retaining functionality, can overcome the cross-species barrier by combining with different types of viral neuraminidase.

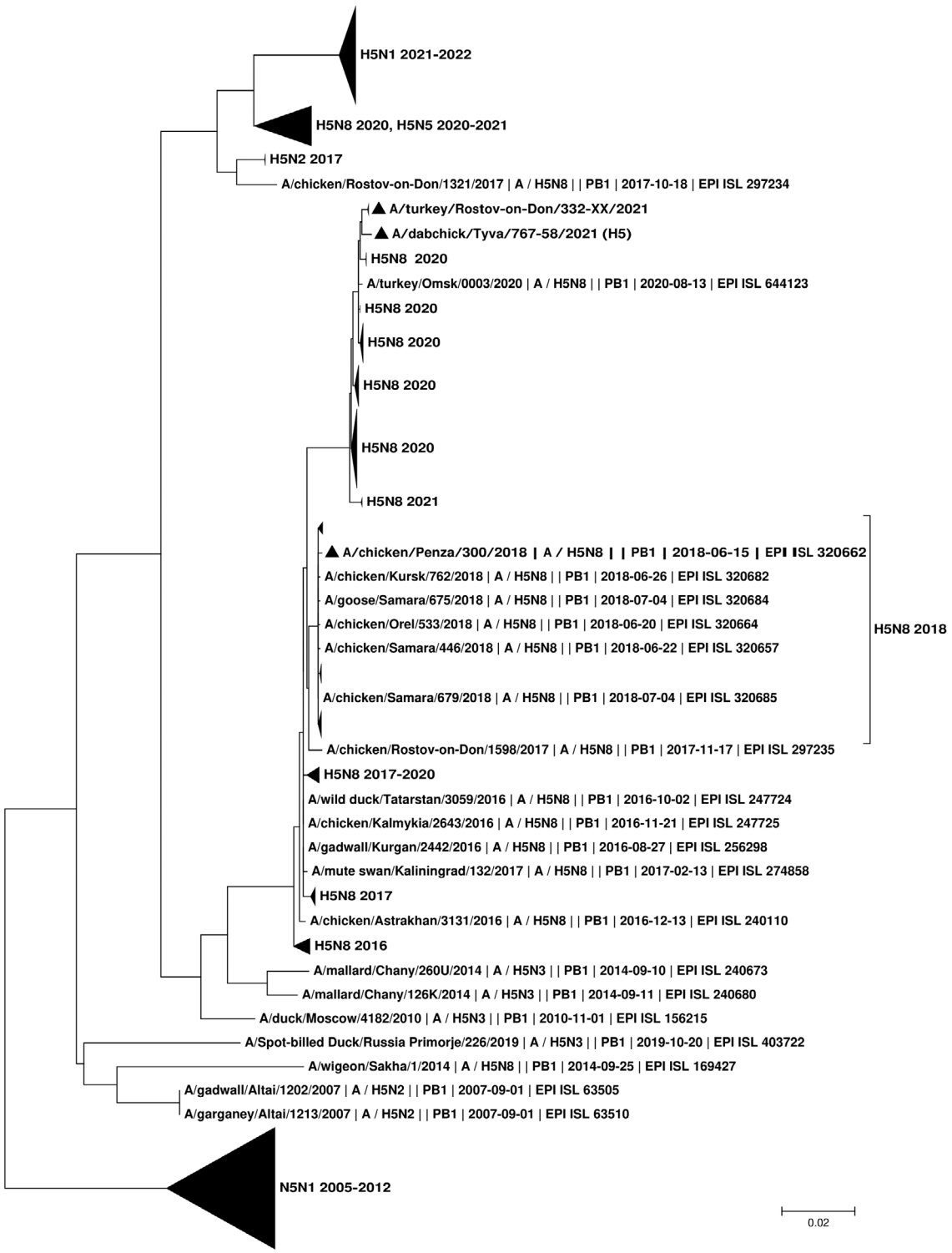
A *678N* mutation was detected in the PB1 protein of HPAI isolated in industrial farming in Rostov region — A/turkey/Rostov-on-Don/332-XX/2021; A/dabchick/Tyva/767-58/2021 virus with *678G* mutation was detected in the territory of the Republic of Tyva. According to experimental data, the combination of amino acids 13P and 678N causes a sharp increase in the polymerase activity of HPAI during replication in mammalian cells [19]. In the A/chicken/Penza/300/2018 isolate, a *105S* mutation was detected that increases the manifestation of virulent properties against mice. To verify the origin of these mutations, phylogenetic analysis was performed, which showed that the A/turkey/Rostov-on-Don/332-08/2021, A/dabchick/Tyva/767-58/2021 and A/chicken/Penza/300/2018 viruses are in groups that include viruses that spread in Russia between 2018 and 2022 and did not have similar mutations (**Figure**). Phylogenetic analysis confirms that the occurrence of mutations *105S*, *678N*, *678G* in the PB1 protein among HPAI detected in Russia is the result of antigenic drift rather than antigenic shifting.

Analysis of the occurrence of substitutions in the replicative proteins of HPAI (as exemplified by mutations *678N* and *105S* of the PB1 protein) showed that the occurrence of single mutations as a result of antigenic drift is extremely rare, even in conditions of frequent epizootics. The single and insignificant spread of mutations in the HPAI population that contribute to the development of the disease in mammals in case of cross-species transmission demonstrates the feasibility and effectiveness of the stepping-out strategy in the control of avian influenza as a disease with zooanthroponotic potential. The use of a stepping-out strategy, involving the total eradication of all infected animals, is recommended by the World Organization for Animal Health for a number of emergent diseases that have panzootic potential and can persist in wildlife populations. With proper surveillance of poultry populations using molecular biology techniques, HPAI can be detected in a timely manner and eradication of the outbreak can be carried out without allowing the possibility of cross-species transmission to mammals. The effectiveness of the stepping-out strategy is confirmed by the disappearance from the viral population of such mutations as *127A* in the PA protein and *292V* in the PB2 protein. These mutations were identified in viruses during the 2018 H5N8 subtype VHP epizootic. The *292V* mutation in the PB2 protein was registered in isolated cases after 2018, but timely eradication of infected stock prevented HPAI from entering the mammalian population. From the HPAI population, these mutations were eliminated by natural selection because they had a negative effect on virus reproduction in birds. Apparently, there is a dynamic equilibrium in HPAI populations, maintained by natural selection, due to which adaptation mutations to mammals disappear from the population. This can be seen in the scattering of such mutations in various genes among HPAI – they occur in different viruses with different frequencies, but no viruses have been found that contain in their genome the full range of adaptation mutations to mammals. In addition, new substitutions in positions, changes in which affect replication in mammalian cells, were identified in the viruses we studied (Table 3).

The effect of new mutations on the replication and virulence properties of HPAI in mammals has not been experimentally studied. Despite the lack of experimental data indicating an increase in virus replication from new substitutions, this is worrisome because early work on the evolution of HPAI showed that, after crossing the species barrier, it undergoes a phase that allows it to gradually acquire adaptive mutations without losing adaptation to the old host [23, 24]. Earlier studies on the transmissibility of HPAI performed by other researchers indicate the complex and often complex nature of changes in the HPAI genome during cross-species transmission and fixation of the virus in the population of a new species [25–28]. Studies carried out at the N.F. Gamaleya Research Center for Epidemiology and Microbiology also showed the possibility of adaptation and acquisition of pathogenic properties for laboratory mice during 7–10 cycles of experimental infection [29].

In our analysis, in addition to single substitutions, it was noted that viruses capable of translating the full-length PB1-F2 protein became established in the post-2020 HPAI population in Russia. Translation became possible due to the nucleotide mutation *A129C* (numbering from the beginning of the open reading frame of the *PB1* gene), which eliminated the stop codon. The PB1-F2 gene is located within the reading frame of the *PB1* gene and encodes a protein that influences the severity of the inflammatory process. At present, there is no unambiguous description of the effect of this protein on the virulence properties of the virus. It is reliably known that the phenotypic expression of the same form of the *PB1-F2* gene differs between birds and mammals. It has already been shown that expression of *PB1-F2* reduces virulence for birds [30-32]. The results of experimental infection revealed that while no virulence enhancement was observed in birds, infection of mice revealed a clear involvement

of the PB1-F2 protein of the H7N1 virus in the host inflammatory response, as previously shown for the H1N1 and H5N1 subtype HPAI strains [32]. The results of experimental infection in ferrets when infected with PB1-F2-expressing HPAI differed from the course of the infection process when the virus without PB1-F2 expression was used. Infection with a virus expressing PB1-F2 correlated with a significant dysregulation of leukocyte counts on days 3 and 7 post-infection; PB1-F2 expression was associated with both lymphopenia and increased neutrophil counts. Lymphopenia was transient in all ferrets, and leukocyte levels



Phylogenetic tree constructed by the NJ method based on the nucleotide sequences of the *PB1* gene fragment (1–2275 bp ORF) of H5 subtype HPAI.

Triangles indicate HPAI with adaptation mutations.

returned to baseline on day 19 post-infection [33]. All viruses with an active form of the PB1-F2 gene have a *66S* amino acid mutation. Several studies have shown that viruses with this mutation caused a more severe infection process in infected laboratory mice [34]. This mutation was present in HPAI isolated from minks in Spain in 2022. [5]. Furthermore, this mutation was one of those that distinguished the deadly virus A/Brevig Mission/18, also known as the "Spanish flu" that swept the world in the early twentieth century [34]. In addition to the direct effect of PB1-F2 protein on the course of the infectious process, data have been obtained that indicate the possibility of a more severe infectious process in case of mutual expression of full-length PB1-F2 and PA-X proteins [35]. The analysis showed that all the HPAI studied in this study are capable of expressing the full-size PA-X protein.

The variability of the C-terminal sequence of the NS1 protein is worth special attention. Earlier studies have shown that single substitutions in the last 4 amino acids affect the possibility of effective cross-species transmission from pigs to mice, accompanied by the manifestation of pathogenic properties in relation to the new host [36]. Thus, H1N1 subtype HPAI that had an NS1 protein with the last 4 amino acids being PEQK and RSEV could not infect mice, whereas the same virus whose NS1 protein ended with the amino acid motif of GSEI and EPEV successfully induced the infectious process in mice, reaching a titer of 2300 BOU/g [37]. Among the HPAI detected in Russia during 2018-2022, the NS1 protein end motif had variations of GSEV, LPPK, FPPK, ESEV and ESEI. Such diversity of the NS1 protein end motif may ensure a wide distribution of HPAI among different bird species [38].

The data obtained by analyzing the presence of marker substitutions in HGP/H5 indicate an active evolutionary process currently taking place in the HPAI population. The presence of mammalian pathogenicity factors in the HPAI population may contribute to successful cross-species transmission of the virus by suppressing certain elements of immune defense. After the cross-species transmission, the virus may end up in the channel of accumulative variability, when in the process of natural selection, single mutations that enhance the phenotypic manifestation or functional properties of certain proteins and provide competitive advantages relative to other viruses are consolidated.

This scenario of cross-species transmission emphasizes the necessity to use the strategy of stepping-out and ban on vaccination against HPAI in industrial poultry production as a deterrent to HPAI as a zooanthroponotic pathogen. Timely and complete elimination of infected poultry stock allows avoiding cross-species transmission of HPAI to mammals via stray dogs, cats or small rodents. In case of uncontrolled vaccination against HPAI, there may be a latent circulation of HPAI among susceptible stock without clinical signs, which will result in an increase in genetic diversity of the HPAI population and active appearance of new mutations, among which may be useful for the virus, contributing to cross-species transmission.

Conclusion

As a result of these studies, it was found that among the H5 subtype HPAI detected in Russia in 2018–2022, viruses with an enzyme complex adapted to replication in avian cells predominated. Adaptation mutations to replication in mammalian cells are sporadic and chaotically distributed in the virus population. Viral hemagglutinin has affinity predominantly to avian cell receptors. The appearance and distribution of virulence factors for mammals in the HPAI population has been shown. The presence of such factors may contribute to successful cross-species transmission of the virus with subsequent adaptation of viral hemagglutinin to mammalian cell receptors as a result of antigenic drift and fixation of new mutations in the course of natural selection.

The results obtained indicate the effectiveness of the strategy of stepping-out and ban on vaccination against HPAI in industrial poultry farming as a deterrent factor for HPAI as a pathogen of zooanthroponotic disease. Timely and complete elimination of poultry stock infected with HPAI, which is capable of producing pathogenicity factors for mammals in the process of reproduction and possessing separate adaptive mutations in replicative proteins, allows to avoid or significantly reduce the probability of cross-species transmission of HPAI from birds to mammals via stray animals or small rodents.

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SCIENCE AND PRACTICE

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Improvement of the bacteriological method for isolation of *Listeria monocytogenes*

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Abstract

Introduction. Currently, listeriosis is regarded as one of the dangerous infections that cannot be prevented by vaccination and is characterized by the severity of the clinical process and high mortality. Improving laboratory diagnostic methods especially in listeriosis meningitis to identify the pathogen in the shortest possible time remains an urgent problem.

The aim of the study was to investigate the behavior of collection and clinical strains of various listeria species on GBM-agar — a nutrient medium for isolating pathogens of purulent bacterial meningitis — in order to improve the bacteriological method for isolating and identifying *Listeria monocytogenes*.

Materials and methods. In the current study, 1125 samples of clinical material and food produces were used. Of these, 95 were isolated and 5 were reference strains of *Listeria* spp. The following culture media to isolate listeria were used: Agar *Listeria* by Ottaviani and Agosti (ALOA); *Listeria* enrichment broth (LEB), *Listeria* isolation agar (LIA), GBM-agar.

Results. Of the 1125 samples involved the following strains were isolated using LEB, LIA and ALOA media: *L. monocytogenes* — 89, *L. welshimeri* — 2, *L. innocua* — 3, *L. seeligeri* — 1. All isolates and reference strains were subcultured by using conventional selective media (LIA, ALOA) and additionally GBM-agar modified by adding a selective additive to isolate *L. monocytogenes*, and yolk emulsion. Colonies grown on the modified GBM-agar and belonging to the *Listeria* genus were larger and had distinctive morphological traits making them differ from colonies obtained by means of conventional listeriosis media. This allowed for the primary differentiation of *L. monocytogenes* from non-pathogenic listeria species and some other pathogens of purulent bacterial meningitis.

Conclusion. It is shown that the algorithm of the culture method can use a new nutrient medium (modified GBM agar) possessing improved growth properties for *L. monocytogenes*, the introduction of which will serve as an additional effective means for differentiating listeria during research in sanitary and clinical microbiology.

Keywords: *listeria, Listeria monocytogenes, nutrient media, modified GBM-agar, food-borne infection*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives.The research protocol was approved by the Ethics Committee of the State Research Center for Applied Microbiology and Biotechnology (protocol No. ВП-2025/3, May 17, 2025).

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Оригинальное исследование
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Усовершенствование бактериологического метода при выделении *Listeria monocytogenes*

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Аннотация

Введение. Листериоз расценивается как одна из опасных вакцинонеуправляемых инфекций, характеризующаяся тяжестью клинического процесса и высокой летальностью. Актуальным направлением остаётся совершенствование методов лабораторной диагностики, особенно при листериозном менингите, для выявления возбудителя в оптимально сжатые сроки.

Цель исследования — изучить поведение музейных и клинических штаммов различных видов листерий на ГБМ-агаре — питательной среде для выделения возбудителей гнойных бактериальных менингитов — для усовершенствования бактериологического метода при выделении и идентификации *Listeria monocytogenes*.

Материалы и методы. В работе использованы 1125 образцов клинического материала и пищевых продуктов, 95 выделенных и 5 референтных штаммов *Listeria* spp., питательные среды для выделения листерий: агар *Listeria* по Оттавиани и Агости (ALOA); питательный бульон для выделения и культивирования листерий (ПБЛ), питательный агар для выделения листерий (ПАЛ), ГБМ-агар.

Результаты. Из 1125 образцов, поступивших на исследование, с использованием ПБЛ, ПАЛ и ALOA выделены штаммы: *L. monocytogenes* — 89, *L. welshimeri* — 2, *L. innocua* — 3, *L. seeligeri* — 1. Все изоляты и тест-штаммы субкультивировали на традиционные селективные среды (ПАЛ, ALOA) и дополнительно на ГБМ-агар, модифицированный внесением селективной добавки для выделения *L. monocytogenes* и желточной эмульсии. На модифицированном ГБМ-агаре выросшие колонии, относящиеся к роду *Listeria*, были крупнее и имели отличительные морфологические признаки от колоний, полученных на классических листериозных средах, что позволило провести первичную дифференциацию *L. monocytogenes* от непатогенных видов листерий и других возбудителей гнойных бактериальных менингитов.

Заключение. Показана возможность использования в алгоритме культурального метода новой питательной среды (модифицированный ГБМ-агар), обладающей улучшенными ростовыми свойствами в отношении *L. monocytogenes*, внедрение которой послужит дополнительным эффективным средством для дифференциации листерий при проведении исследований в санитарной и клинической микробиологии.

Ключевые слова: *листерии, Listeria monocytogenes, питательные среды, модифицированный ГБМ-агар, пищевая инфекция*

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Introduction

Listeriosis is not only a medical and social but also an economic problem. Despite the fact that in recent years the incidence of listeriosis has been kept at the level of sporadic cases, listeriosis is considered as one of the dangerous vaccine-uncontrollable infections, characterized by the severity of the clinical process and high mortality (up to 20%) [1, 2].

The incidence of listeriosis is caused by contamination and active multiplication of listeria in foodstuffs, increased susceptibility to listeria in risk groups against the background of cellular immunity disorders [1, 3]. In recent years, data have been obtained on the circulation of Listeria in plant, soil, and water substrates, their high adaptive capabilities in a wide temperature range, humidity, and pH of the environment. There is data on the contamination of water sources with the causative agent of listeriosis near livestock enterprises [4].

In healthy people, infection is usually asymptomatic or in the form of gastroenteritis [1, 2, 5]. In the immunocompromised elderly, pregnant women and newborns, or in patients receiving immunosuppressive therapy, listeriosis may manifest as bacteremia or sepsis, central nervous system involvement, *etc.*, leading to serious or potentially fatal illnesses, including sepsis or meningitis [2, 6, 7–12].

The clinical manifestations of these forms of the disease, including listeriosis meningitis, are nonspecific, mainly fever, headache, vomiting, and disorders of consciousness, which is similar to other types of purulent meningitis [2, 3, 6, 7].

Listeriosis is caused by gram-positive, facultatively anaerobic, enteroinvasive bacteria of the genus *Listeria.* The main causative agent of the disease in humans *is L. monocytogenes*, which is capable of causing listeriosis in animals [19]. The main causative agent of listeriosis in animals is *L. ivanovii*, which in rare cases can lead to the disease in humans [20]. There are isolated cases of listeriosis caused by *L. innocua* and *L. seeligeri* [21, 22].

In the laboratory diagnosis of listeriosis and sanitary-bacteriologic investigations, the leading role is played by the bacteriologic method using nutrient media [13].

The isolation of Listeria from non-sterile clinical material and foodstuffs is only possible using selective nutrient media or enrichment procedures. Listeria enrichment broth (LEB), Fraser broth, UVM broth are used; as selective differential diagnostic media, Listeria isolation agar (LIA), Listeria agar by Ottaviani and Agosti (ALOA), Brilliance Listeria agar, Oxford agar, PALCAM agar, etc. are used as selective enrichment media.

Among the various media, LIA and PALCAM agar media do not provide species differentiation of *Listeria*. On such media, the isolation of *Listeria* is based on their ability to hydrolyze esculin to form esculentine, which in the presence of iron ions forms a black complex; as a result, *Listeria* of all species form grayish colonies with a black zone around them.

Chromogenic media (ALOA and Brilliance Listeria agar) with special selective and chromogenic additives allow to isolate *Listeria* of different species in the form of blue-green colonies and differentiate *L. monocytogenes* and certain strains of *L. ivanovii* from other *Listeria* species by formation of a characteristic halo around the colonies due to the ability to produce phospholipase C.

The selectivity of the media with respect to associated microflora is ensured by the inclusion of lithium chloride, acriflavine, cycloheximide, nalidixinic acid, polymyxin and other antibiotics [14].

For the isolation of *Listeria* from normally sterile biological substrates (blood, cerebrospinal fluid, etc.), which is typical in the bacteriological study of meningitis, blood and chocolate agar can be used, as well as GBM-agar, a nutrient medium for the isolation and cultivation of agents of purulent bacterial meningitis without selective additives [15]. *Listeria* on these media grows as round convex translucent non-pigmented colonies with a smooth surface after 24–48 h of cultivation.

The rich GBM-agar base containing casein hydrolysate, peptone, yeast extract, growth stimulator of hemophilic microorganisms, and glucose is able to support the growth of *Listeria* of various species.

Since the main purpose of GBM-agar is related to the cultivation and isolation of the three main pathogens of bacterial meningitis: *Haemophilus influenzae* type B, *Streptococcus pneumoniae*, *Neisseria meningitidis*, it contains selective additives only for them. Selective additives for *Listeria* have a different composition of antibiotics and contain the dye acriflavine, which has antiseptic properties and can give *L. monocytogenes* colonies a green color.

Preliminary studies of clinical material from meningitis patients have shown that the use of GBM-agar with a selective additive for *Listeria* allows isolation of the listeriosis pathogen in a shorter time than conventional listeria media.

To introduce the nutrient medium into the scheme of laboratory diagnostics of listeriosis, studies using a wide range of strains belonging to *L. monocytogenes* and other *Listeria* species are necessary.

The aim of the study is to investigate the behavior of reference and clinical strains of different *Listeria* species on GBM agar to improve the bacteriological method for the isolation and identification of *L. monocytogenes* in clinical and sanitary microbiology.

Materials and methods

The following materials were used: clinical material (CSF, sectional material, blood, cerebrospinal fluid, cervical canal secretion) and food raw materials and

food products received by the testing laboratory center of the State Research Center for Applied Microbiology and Biotechnology (SRC AMB) (a total of 1125 samples); reference strains of microorganisms obtained from the State Collection of Pathogenic Microorganisms and Cell Cultures "SCPM-Obolensk": *L. monocytogenes* 766, *L. monocytogenes* NCTC11994, *L. ivanovii* ATCC19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *Escherichia coli* ATCC 25922, Proteus vulgaris HX 19 222, *Staphylococcus aureus* Wood-46, *S. pneumoniae* ATCC 6305, *N. meningitidis* ATCC 13102, *H. influenzae* ATCC 49247. Sample preparation and research were carried out using algorithms and methods recommended by SanPiN 3.3686-21[1], MG 4.2.1122-02[2], GOST 32031-2022[3]. The study was conducted with the informed consent of the patients or their legal representatives.The research protocol was approved by the Ethics Committee of the SRC AMB (protocol No. ВП-2025/3, May 17, 2025).

Nutrient media produced by SRC AMB were used for Listeria accumulation: nutrient broth for cultivation and isolation of *Listeria* with selective additive (LEB medium) FSR 2010/09161; as differential-diagnostic — nutrient agar for cultivation and isolation of Listeria with selective additive (ALOA medium, SRC AMB, RU No. FSR 2010/09162); Listeria Ottaviani Agosti HiCynth Agar (ALOA, HiMedia, RU No. FSZ 2009/03705) with Enrichment Supplement (FD214), Selective Supplement (FD 212A); nutrient medium for isolation of purulent bacterial meningitis pathogens (GBM-agar, State Research Center AMB, RU No. RZN 2016/4872) with selective additive (polymyxin B sulfate — 0.01 g/L; nalidixic acid — 0.025 g/L; acriflavine — 0.01 g/L) (hereinafter — modified GBM-agar).

To determine lecithinase activity we used nutrient medium for quantitative determination of microbial contamination (medium No. 1 GRM, SRC AMB, RU No. FSR 2011/11415) and modified GBM-agar with ex tempore addition of 5% yolk emulsion, and the same media with the addition of activated carbon at a concentration of 0.5%.

Identification of isolates was performed on a MALDI Biotyper automatic system (Bruker Daltonik).

Results

The studies were conducted in 2 stages. The first stage was devoted to the isolation of listeriosis pathogen from clinical material, food raw materials and food products. The samples prepared for the study were seeded on enrichment medium — LEB. After 24 and 48 h of incubation at 37 ± 1°C from LEB medium, culture fluid was seeded on special nutrient media for the isolation of Listeria (LIA, ALOA). After incubation, characteristic colonies, presumably belonging to Listeria, were selected and subcultured on GRM medium No. 1 for further identification using a MALDI Biotyper automatic system. During the study, 89 isolates of *L. monocytogenes*, 2 isolates of *L. welshimeri*, 3 isolates of *L. innocua*, and 1 isolate of *L. seeligeri* were obtained.

At the second stage, we studied the behavior of all isolates, including those from meningitis patients, and test strains of Listeria on GBM agar in comparison with their behavior on classical nutrient media (LIA, ALOA) under conditions of equivocation.

On LIA medium, isolated cultures and reference strains of *L. monocytogenes* formed small, grayish colonies up to 1.0 mm in diameter after 24 h; on the 2nd day their size increased to 1.2–1.4 mm. On ALOA medium, *L. monocytogenes* grew as blue-green colonies surrounded by an opaque halo 0.6-0.8 mm in diameter on the 1st day of incubation and up to 2.0 mm in diameter on the 2nd day. On modified GBM-agar already after 18 h of *L. monocytogenes* incubation, the diameter of colonies reached 1.5–2.5 mm (**Fig. 1**). The colonies acquired a greenish color, in contrast to the colonies obtained on the classical LIA medium.

The growth of other *Listeria* species on GBM agar differed from that of *L. monocytogenes*. When studying their growth character, museum test strains: *L. ivanovii* ATCC19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967 and isolated cultures of Listeria species: *L. welshimeri*, *L. innocua*, *L. seeligeri* were sown on modified GBM-agar and comparison media LIA and ALOA. After 18–24 h of incubation on the modified GBM-agar, the growth of these Listeria species was detected in the form of smooth milk-colored colonies with a diameter of 1.6–2.0 mm, in contrast to LIA and ALOA media, on which the growth of small colonies with a diameter of 0.3–0.8 mm was observed.

When seeding from a mixture of reference strain *L. monocytogenes* 766 and *L. innocua* NCTC 11288 on modified GBM agar and subsequent incubation at 37 ± 1°C for 18 h, the medium was found to have differentiated properties: the growth of reference strain *L. monocytogenes* 766 was observed as greenish colored colonies with a diameter of 1.8–2.2 mm; *L. innocua* NCTC 11288 — as milky colored colonies with a diameter of 1.5–1.8 mm (**Fig. 2**).

Furthermore, the addition of yolk emulsion to the modified GBM-agar medium provided a clear differentiation of the reference strain *L. ivanovii* ATCC19119, which has lecithinase activity, from the reference strain *L. innocua* NCTC 11288, which does not have lecithinase (**Fig. 3**).

Studies of clinical material, food raw materials and food products for the presence of *Listeria* include

[1] SanPiN 3.3686-21 Sanitary and epidemiologic requirements for the prevention of infectious diseases" (enacted on 01.09.2021).

[2] MG 4.2.1122-02 Organization of control and methods of detection of *Listeria monocytogenes* bacteria in food products: methodological guidelines. Moscow; 2002.

[3] GOST 32031-2022 Food products. Methods of detection of bacteria *Listeria monocytogenes* and other *Listeria* species (*Listeria* spp.).

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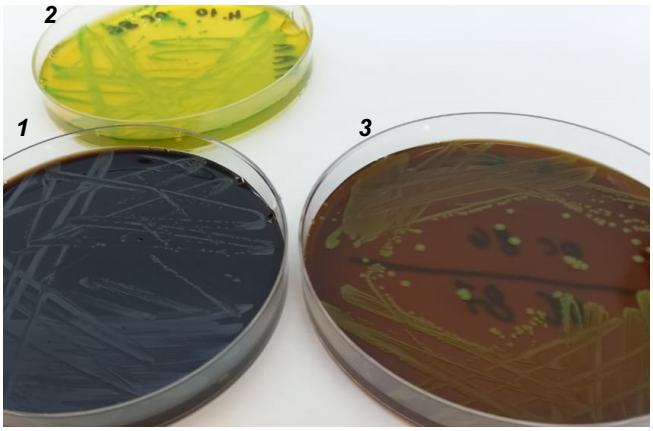

Fig. 1. Growth of isolated *L. monocytogenes* cultures on LIA (*a*), ALOA (*b*) and modified GBM agar (*c*).

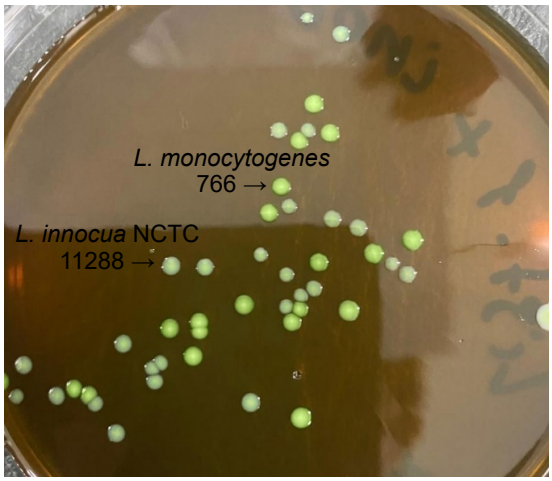

Fig. 2. Growth of a mixture of reference strains of *L. monocytogenes* 766 and *L. innocua* NCTC 11288 on modified GBM agar.

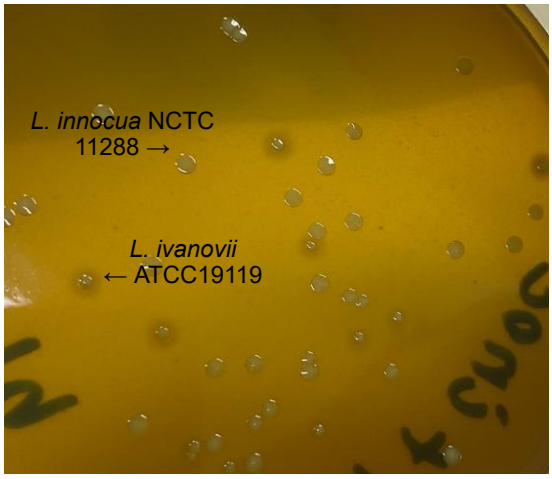

Fig. 3. Growth of a mixture of reference strains of *L. ivanovii* ATCC19119 and *L. innocua* NCTC 11288 on modified GBM agar with yolk emulsion addition.

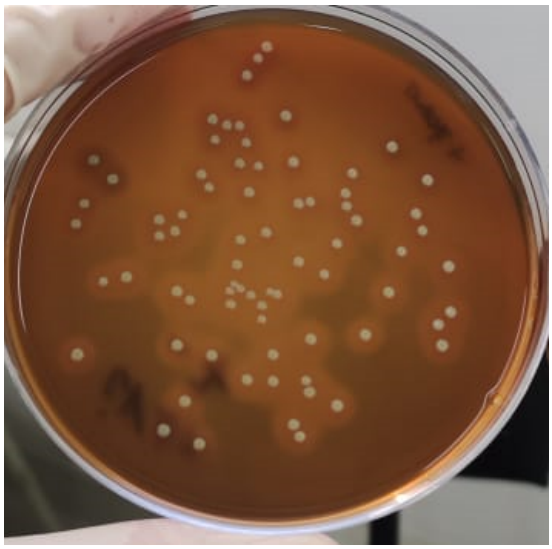

Fig. 4. Growth of reference strain *L. ivanovii* ATCC19119 on modified GBM-agar medium with yolk emulsion addition.

a stage to determine the lecithinase activity, the presence of which is an indicator of pathogenicity, to confirm the affiliation of isolated bacteria to the species *L. monocytogenes*. The originality of the bacteriological method is based on the comparison of lecithinase activity of the culture in the presence or absence of activated carbon. In this study, an attempt was made to determine the lecithinase activity of Listeria in accordance with the method regulated by the regulatory documents MG 4.2.1122-02 and GOST 32031-2022 using modified GBM-agar with the addition of yolk emulsion with and without activated carbon. The isolated cultures of *L. monocytogenes* and reference strains *L. monocytogenes* 766 and *L. ivanovii* ATCC19119 were seeded on medium from dilutions 10^{-6}. Seeds were incubated at 37 ± 1°C for 24 h and viewed under transmitted light. The traditionally used for this purpose medium No. 1 GRM medium was used as a control medium of comparison.

On medium No. 1 GRM with the addition of yolk emulsion in the presence of charcoal after 24 h, a dense zone of turbidity around the colonies of isolated strains of *L. monocytogenes*, reference strain *L. monocytogenes* 766 and *L. ivanovii* ATCC19119 with a width of more than 2.0 mm, characteristic of the lecithinase activity of cultures, was observed. On the same medium without activated charcoal, no zones of opacity were observed around *L. monocytogenes* colonies, in contrast to *L. ivanovii* ATCC19119 colonies.

On modified GBM-agar with yolk emulsion without charcoal, a clearly distinguishable lecithinase activity was also observed only around *L. ivanovii* ATCC19119 colonies (**Fig. 4**).

Addition of activated carbon to the modified GBM-agar in the same concentrations resulted in more intense blackening of the medium, which did not allow us to consider and correctly interpret the results of lecithinase activity detection.

When determining the selective properties of modified GBM-agar, growth suppression of reference

strains of *E. coli* ATCC 25922, *P. vulgaris* HX 19 222, *S. aureus* Wood-46, *S. pneumoniae* ATCC 6305, *N. meningitidis* ATCC 13102, *H. influenzae* ATCC 49247 from dilutions 10^{-4} was detected.

Discussion

Meningitis caused by *L. monocytogenes* is a serious and life-threatening disease. In recent years, *L. monocytogenes* is the third most common cause of bacterial meningitis in the elderly, after *S. pneumoniae* and *N. meningitidis*, because of a decrease in the incidence of meningitis caused by *H. influenzae* type B due to vaccination [18]. The disease is more common in elderly people, clinical manifestations in this type of meningitis are not specific, so for the correct diagnosis it is important to take into account the results of bacteriological examination aimed at isolation and identification of the pathogen.

The GBM-agar nutrient medium designed for isolation of the main pathogens of purulent bacterial meningitis has proven itself in the study of clinical material for the presence of *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* type B [15]. Therefore, it was of interest to study the possibility of using this nutrient medium for the isolation of *Listeria*, but using a special selective additive for *Listeria*. The antibiotics polymyxin B, nalidixic acid, ceftazidime, and acriflavine included in the medium inhibit the growth of the main pathogens of GBM, as well as a number of other microorganisms (e.g., *E. coli*, *P. vulgaris*, *S. aureus*). The medium with the selective additive is called modified GBM agar.

In our study, we examined the behavior of different *Listeria* species on modified GBM agar: 89 isolates of *L. monocytogenes*, 2 isolates of *L. welshimeri*, 3 isolates of *L. innocua*, 1 isolate of *L. seeligeri*, and 5 reference strains of *Listeria* spp. On modified GBM-agar, *L. monocytogenes* colonies acquired a greenish color and after 18 h of incubation significantly exceeded the colonies on the classical LIA medium and even ALOA. At the same time, non-pathogenic *Listeria* formed small colonies of milky/cream color. This ability of modified GBM-agar will accelerate the isolation and differentiation of the main listeriosis pathogen — *L. monocytogenes* — from other *Listeria* species by morphological features of colonies (color and size).

Addition of yolk emulsion to modified GBM-agar provided clear differentiation of the test strain *L. ivanovii* ATCC19119, possessing lecithinase activity, from the reference strain *L. innocua* NCTC 11288, lacking lecithinase. However, the addition of activated charcoal, which activates the manifestation of lecithinase activity in *L. monocytogenes* on GRM medium No. 1, no zones of turbidity around *L. monocytogenes* colonies were observed. Perhaps, this situation is explained by more intensive blackening of GBM-agar, which is already colored dark brown due to the growth stimulant of hemophilic microorganisms included in its composition. And on such a dark nutrient medium it is difficult to consider and correctly interpret the results of lecithinase activity detection.

I.S. Tartakovsky and coauthors noted that identification of *L. monocytogenes* by lecithinase activity detection is often difficult due to morphological and biochemical features of the listeriosis pathogen [13]. In the studies of domestic and foreign authors it is mentioned that when cultured on nutrient media containing yolk emulsion, the lecithinase activity of *Listeria* is detected extremely weakly or is not observed at all. The addition of activated carbon can result in the sorption of the product secreted by *Listeria* that inhibits the production of lecithinase enzyme [13, 16, 17].

Conclusion

The possibility of using a new nutrient medium (modified GBM agar) with improved growth properties against *L. monocytogenes* in the algorithm of the culture method is shown. Its implementation will serve as an additional effective means for differentiating *Listeria* during research in sanitary and clinical microbiology.

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REVIEWS

Review
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Organoid (3D-cell) cultures in the assessment of cross-species virus transmission

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Abstract

The **aim** of this review is to characterize the possibilities of using organoid (3D-cell) cultures to assess the ability of viruses for cross-species transmission.

Sources from Web of Science, PubMed, Scopus, Elsevier, Google Scholar, and eLIBRARY.RU databases as of February 2025 were used.

In addition to classical methods of epidemiologic diagnostics and surveillance of viral infections, molecular genetic technologies (polymerase chain reaction and sequencing) are widely used in the epidemiologic surveillance system. As the best world experience shows, the use of organoid (3D-cell) cultures is promising in addressing these issues. This review analyzes data on the use of organoid (3D-cell) cultures of human and animal origin to study immunopathogenesis, as well as to assess the ability of a number of viruses (SARS-CoV-2, influenza, Zika, measles, etc.) for cross-species transmission, which determines their pandemic potential

Keywords: *review, organoid (3D-cell) cultures, viruses, cross-species transmission, epidemiology*

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Научный обзор
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Органоидные (3D-клеточные) культуры в оценке способности вирусов к межвидовым переходам

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Аннотация

Цель обзора — охарактеризовать возможности применения органоидных (3D-клеточных) культур для оценки способности вирусов к межвидовым переходам.

Использованы источники из баз данных Web of Science, PubMed, Scopus, Elsevier, Google Scholar и eLIBRARY.RU по состоянию на февраль 2025 г.
В работе системы эпидемиологического надзора, помимо классических методов эпидемиологической диагностики и надзора за вирусными инфекциями, широко применяются молекулярно-генетические технологии (полимеразная цепная реакция и секвенирование). Как показывает передовой мировой опыт, в решении этих вопросов перспективным является использование органоидных (3D-клеточных) культур. В настоящем обзоре проанализированы данные по применению органоидных (3D-клеточных) культур человеческого и животного происхождения для изучения иммунопатогенеза, а также оценки способности ряда вирусов (SARS-CoV-2, гриппа, Зика, кори и др.) к межвидовым переходам, что обусловливает их пандемический потенциал.

Ключевые слова: *обзор, органоидные (3D-клеточные) культуры, вирусы, межвидовые переходы, эпидемиология*

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Introduction

In order to successfully carry out the tasks that ensure sanitary and epidemiological well-being of the population, the activities of the Russian network of specialized institutions in the field of epidemiological surveillance must be improved upon, which involves a system of comprehensive surveillance of the epidemic process of a particular disease in dynamics in a certain territory in order to improve the effectiveness of preventive and anti-epidemic measures [1].

The COVID-19 pandemic, etiologically associated with SARS-CoV-2 (severe acute respiratory syndrome coronavirus type 2) (Nidovirales: Coronaviridae, *Betacoronavirus*, *Sarbecovirus* subgenus), on the one hand, demonstrated the predictive capabilities of epidemiologic methods (local specialists had warned about the pandemic potential of coronaviruses several years before the pandemic [2-4]), and on the other hand, contributed to the formation of new approaches, the main one being genomic epidemiologic surveillance [5]. Genomic epidemiologic surveillance is currently one of the main elements of the large federal project "Sanitary Shield – Safety for Health (Prevention, Detection, Response)", which Rospotrebnadzor is implementing in the territory of the Russian Federation [6]. This project includes the construction of a network of diagnostic PCR laboratories and Sequencing Centers equipped with modern highly efficient NGS sequencers [5, 7]. The obtained data are aggregated by the Russian genetic data platform VGARus [5]. However, along with molecular genetic methods of research, classical methods of epidemiologic surveillance in the field of virology, in particular cell culture approaches, also continue to be actively developed [8, 9]. At the same time, 3D-cell cultures have been utilized in response to current necessities and opportunities.

The ability of viruses for cross-species transmission is responsible for the pandemic potential of viruses [10-12]. Most pandemics are associated with viruses, which may include unidentified and potentially dangerous viruses capable of rapid evolution and transmission from one host organism to another. The emergence of new potentially dangerous viruses may be caused by climate change and the melting of polar glaciers, urbanization and wildlife trade. Due to the possibility of new epidemic outbreaks of viral diseases caused by new or mutating viruses that pose significant threats to public health, it is necessary to plan adequate preventive and anti-epidemic measures. These include the development of new experimental models for studying viruses. Predicting the ability of viruses for cross-species transmission also requires new experimental models.

The aim of this review is to characterize the current possibilities of using organoid (3D-cell) cultures to assess the ability of viruses for cross-species transmission.

The analysis included scientific literature presented in the main databases (Web of Science, PubMed, Scopus, Elsevier, Google Scholar and eLIBRARY.RU) as of February 2025.

Application of organoid (3D-cell) cultures in virology

The method of organoid (3D-cell) cultures has found wide application in virology for culturing and studying the reproduction of human and animal virus-

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es, studying the mechanisms of immunopathogenesis, developing and testing antiviral drugs and vaccines [13–15]. A relatively new aspect of the use of organ cultures is the solution of several issues related to epidemiologic surveillance and monitoring for viral infections. In particular, organoid (3D-cell) cultures are used to assess the ability of viruses for cross-species transmission, which accounts for their pandemic potential.

Organoids and their variant, spheroids, are related to 3D-cell cultures. There is no single definition of organoids, but their integral characteristics are multicellularity, ability to self-organize and perform any physiological functions of an organ. Spheroids are 3D-cell cultures that form sphere-like formations during proliferation, which allows cells to grow and differentiate in several directions.

Organoids are obtained from pluripotent stem cells, including induced pluripotent stem cells and embryonic stem cells, as well as from differentiated cells or tumor tissue cells [16±18]. Protocols for obtaining organoids according to the method are divided into two large groups:

- various modifications of Lancaster's (2014) method [19], differing in terms of cultivation time and the use of differentiation inducers followed by aging in microtubes or plates [20];
- use of bioreactors or mini-bioreactors [21].

One of the approaches to creating 3D-cell models is a cell line grown on a three-dimensional gel framework, as well as a spheroid culture or an organotypic (organ) culture obtained directly by fragmenting an organ and its subsequent cultivation [18]. 3D-cell cultures occupy a more advantageous position compared to 2D-cell cultures and *in vivo* models, as they allow to reproduce the structure of real organs, control signaling pathways and edit cell genomes in an environment resembling an organism, but are deprived of a number of disadvantages of living systems. The different cell morphology in 2D-cell culture from native cells negatively affects cellular processes including proliferation, differentiation, apoptosis, gene expression. Furthermore, these cultures are tumorigenic, genetically unstable, and do not reproduce the complex intercellular interactions required to model viral infections [8, 9, 22].

The advantage of 3D-cell systems, and organoids in particular, over cell lines is the reproducibility of cell layers and tissue structure present in organs. Organoids can be cultured for longer periods, frozen and used to study physiological phenomena more realistically than is possible with cell lines. Although organoid cultures are expensive and difficult to replicate the scale of massive cell line culture systems, technologies to construct them are rapidly advancing [13–15, 23].

The structure of organoids enhances viral tropism to tissues and increases the likelihood of viral infection [14, 23]. Human organoids have become an important tool in the field of viral infections research, and have played a major role in their modeling and investigation of the molecular mechanisms underlying their pathogenesis. Only organoid systems allow us to study the actual virus-host interaction, pathogenesis of infection, treatment and prevention issues. Organoids also represent an indispensable model for cross-species testing of new viruses [14, 23, 24], as discussed below. However, in terms of virology, the use of organoids in Russia is still in its early stages.

As noted by several authors, there is an urgent necessity to use more advanced biological systems for the study of viral infections, including the assessment of the cross-species potential of anthropozoonotic viruses [12, 25].

Application of human organoid cultures to study the ability of viruses to overcome the cross-species barrier

Virus-host interactions are the main driving force behind virus evolution. The ecology of a virus can only be understood through the ecology of its actual and potential hosts [26]. Natural focal pathogens of infectious diseases are co-evolved with natural biocenoses and can circulate without human involvement [27]. If a person finds himself in the territory of a natural focus, they may become an accidental host of the pathogen, and in some cases an anthroponotic chain of its transmission may be formed [28].

A key element of the ecological plasticity of natural focal viruses is their ability to overcome cross-species barriers. This ability is most pronounced in arboviruses, which are transmitted to vertebrates by arthropod vectors [26, 28]. The ecological group of arboviruses includes, in particular, Zika virus (Amarillovirales: Flaviviridae, *Flavivirus*), which causes sporadic morbidity in Africa and Asia, and in 2015 entered South America [29]. Brain organoids have been used to study the immunopathogenesis of Zika fever and routes of transmission of this virus, as it is associated with an increased risk of neurological complications in adults and children, and also causes brain malformations in fetuses of infected pregnant women [30]. Organoid cultures are actively used to study infection pathways and mechanisms of cross-species transmission for other arboviruses: Chikungunya (Martellivirales: Togaviridae, *Alphavirus*), Japanese encephalitis (Amarillovirales: Flaviviridae, *Flavivirus*), Powassan (Amarillovirales: Flaviviridae, *Flavivirus*); Dengue (Amarillovirales: Flaviviridae, *Flavivirus*) [14, 31].

An example of the use of organoids as a suitable model for cross-species virological studies is the discovery and characterization of CD46 cellular receptors for measles virus (Mononegavirales: Paramyxoviridae, *Morbillivirus*). Using a model of human respiratory system organoids, it was found that vaccine and laboratory adapted strains of this virus use CD46 (an adhesion

molecule expressed by human nuclear cells that acts as a costimulatory factor for T-lymphocytes) as a receptor, whereas vaccine and clinical wild-type strains are incapable of using CD46 [32]. Then, another receptor for adhesion of wild-type measles virus strains, nectin-4 (nectin-4) or PVRL4 (poliovirus receptor-related 4), intensively expressed on the basolateral side of epithelial cells, was identified [33]. This discovery led to a new paradigm on how measles virus enters the respiratory tract and leaves the host, with implications for the development of preventive measures.

As for contact-transmitted viruses, the threshold for entry into the human population is lower for great ape viruses (Primates: Hominidae). Human immunodeficiency virus types 1 and 2 once evolved from monkey immunodeficiency virus (Ortervirales: Retroviridae, *Lentivirus*) [28], and the tissue tropism of these viruses is being actively studied on organoid cultures [34]. Monkeypox virus (Chitovirales: Poxviridae, *Orthopoxvirus*), which is of great concern to epidemiologists, is capable of easy transmission from primates to humans and causing epidemic outbreaks [28]. Y. Watanabe et al. (2023) used models of colon organoids and human keratinocytes derived from pluripotent cells to study the replication dynamics of this virus in the respective tissues; it was shown that the virus accumulated most intensively in keratinocytes, whose dysfunction was associated with significant mitochondrial damage [35]. The more unique the primary host of the virus is (not only genetically but also physiologically), the more difficult it is for the virus to overcome the cross-species barrier. Especially interesting in this respect are bats (*Chiroptera*), whose physiology and parasite (including virome) are very specific [36]. Bats are considered a natural reservoir for a variety of viruses, including SARS-CoV-2, Ebola virus and possibly others. In most cases, bat-borne viruses require an intermediate host for effective entry into the human population. The most famous exception to this rule is the rabies-causing lyssaviruses (Mononegavirales: Rhabdoviridae, *Lyssavirus*), which easily overcome cross-species barriers due to the versatility of the nicotinic acetylcholine receptor of nerve endings used for entry of these viruses into the target cell [28]. For this reason, organoids of the nervous system are convenient and widely used 3D-cell models to study infection pathways and mechanisms of lyssavirus cross-species transmissions [37]. For Ebolaviruses (Mononegavirales: Filoviridae, *Ebolavirus*) and Marburgviruses (Mononegavirales: Filoviridae, *Marburgvirus*) associated with hemorrhagic fevers, primates act as facultative (in some cases, direct transmission of the pathogen to humans from mammals is also possible) intermediate hosts, which have long been considered a natural reservoir of filoviruses [38]. In this case, organoids of blood vessels were effective in establishing the specific features of the pathogenesis of infection [39].

For SARS-CoV[1], which caused a major epidemic in China in 2002-2003 [3], the intermediate host was Himalayan civets (*Paguma larvata*) [40]; for MERS-CoV (Middle East respiratory syndrome coronavirus), which caused a series of epidemic outbreaks in the Arabian Peninsula and many imported cases worldwide [3], the intermediate host was one-humped dromedary camels (*Camelus dromedarius*) [40]; for pandemic SARS-CoV-2, pangolins (*Pholidota*), which are widely found in Southeast Asian markets because their derivatives are used in Oriental medicine and their meat is considered a delicacy [40, 41]. The significant epidemic potential of bat-borne coronaviruses [40] necessitates the development of organoid models to study the cross-species transmission of these viruses.

Given that influenza, Ebola, Zika and pandemic coronavirus (SARS-CoV-2) viruses have demonstrated significant public health threats, organoid cultures have found applications in better understanding the mechanisms of pathogenesis and routes of infection in these infections.

In parallel with the widespread implementation of genomic surveillance, the COVID-19 pandemic has stimulated the implementation of 3D-cell models to study the pathogen of this disease, in particular, organoid cultures of human lungs, bronchi and tonsils, liver and intestines, kidneys and blood vessels [42]. COVID-19 has been shown to be a vascular disease and cause direct damage to the endothelium [43]. The neuroinvasive potential of SARS-CoV-2 has been investigated on brain organoids [44]. The use of human intestinal enteroids in which sustained replication of SARS-CoV-2 was maintained, along with the detection of viral RNA in fecal samples and the development of gastrointestinal symptoms in some COVID-19 patients, confirmed that the gastrointestinal tract may serve as one of the routes of SARS-CoV-2 transmission in addition to airborne transmission [45]. The use of an organoid model of the human upper respiratory tract and lungs to culture SARS-CoV-2 has shown that this relevant and reliable model for coronavirus research has additional value for testing other respiratory viruses, studying immunopathogenesis, and developing therapeutic and preventive measures [42].

One of the most studied examples of a virus overcoming cross-species barriers is the influenza A virus (Articulavirales: Orthomyxoviridae, *Alphainfluenzavirus*), whose natural reservoir is birds of the aquatic-ecological complex, primarily geese (*Anseriformes*) and plovers (*Charadriiformes*) [26-28]. All variants of this virus circulating among mammals, including epidemic [46] and pandemic [47] variants, have precursors in wild bird populations.

[1] Due to the emergence of SARS-CoV-2, it is now acceptable to refer to SARS-CoV as SARS-CoV-1

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Brain organoids have been used to study the pathways of infection during infection caused by influenza A virus subtypes H1N1, H3N2, H7N1, and H5N1 [14, 48]. Organoids of the human respiratory tract with ciliated epithelium have also been used to study the multiplication ability of influenza viruses and other respiratory infections [48, 49]. For example, bronchial organoids have been used to culture influenza viruses of types A (*Alphainfluenzavirus*), B (*Betainfluenzavirus*), and C (*Gammainfluenzavirus*) [50]; lung organoids have been used to culture parainfluenza viruses (Mononegavirales: Paramyxoviridae) of types 1, 3 (*Respirovirus*), 2, 4 (*Rubulavirus*) [48]. Human respiratory tract organoids containing the main types of epithelial cells of the respiratory tract have shown different degrees of infectivity of human and avian strains in influenza A modeling. This relates to virus multiplication, tropism to tissues and cytokine production on these strains [51].

Using organoid models of the respiratory tract, the receptor-binding site of hemagglutinin of strains adapted to birds was studied. It was found that this site has a high affinity for α2'-3'-sialosides, while epidemic strains have affinity for α2'-6'-sialosides; pigs (*Suidae*) contain cells with both of these types of sialosides, so natural adaptation of avian variants of the virus to human receptor specificity may occur in their organism [26, 28, 52]. The situation is complicated by the fact that the cells of the columnar epithelium in the upper parts of the human respiratory tract carry mainly α2'-6'-sialosides on their surface, and in the lower parts, they carry α2'-3'-sialosides. Therefore, during infection of each individual human organism, a gradual positive selection of viral variants with α2'-3'-specificity of the hemagglutinin receptor-binding site is possible as the infection passes from the upper respiratory tract to the bronchioles, which contributes to the development of severe (up to lethal) primary viral pneumonias [53]. In this regard, the development of organoid (3D-cell models) to study the drift of receptor specificity of influenza A virus depending on the conditions of its interaction with cells is important not only in the context of virus adaptation to the human body and to study the issues of overcoming the cross-species barrier by viruses, but also to predict the clinical consequences of the development of infection.

Animal organoid cultures in the study of viruses overcoming the cross-species barrier

According to various estimates, of the 1,500 known infectious diseases in the world, 60% are of animal origin, with about 75% of new infectious diseases being zoonotic in nature, and 25% of zoonoses occurring in domestic animals. Viruses are etiologic agents of zoonoses in about 30% of cases. Zoonotic viral infections in animals are direct evidence of the ability of viruses to overcome cross-species barriers and infect humans [41, 54, 55].

The use of animal organoids for modeling zoonotic infections opens prospects for the study of host-pathogen interactions in zoonotic viral infections [41, 56, 57]. In this aspect, in addition to molecular genetic methods, cross-species organoid cultures based on human and animal cells are of considerable interest for scientifically justified prediction of the emergence of new viral variants dangerous for humans with epidemic potential [58]. According to several researchers, the use of such organoids helps to provide a biosystem to confirm the zoonotic potential of newly emerging viruses, to effectively study the infection cycle of these viruses in different species of domestic and wild animals and the ability of viruses to cross-species transitions, including adaptation to the human body. Furthermore, the use of cross-species organoids allows the cultivation of new viruses that cannot be grown in cell lines [57, 58].

Y. Sang et al. analyzed the status and potential of cross-species organoid cultures and noted the necessity for their development to study cross-species susceptibility and investigate newly emerging zoonotic viruses in both domestic and wild animals. The authors also noted the necessity to adapt the technology of human organoid production for the development of animal organoids, and in the 1st place, based on respiratory organs [58].

Despite intensive research due to the spread of the COVID-19 pathogen and other zoonotic respiratory viruses, there have been no reports of animal respiratory or pulmonary organoids until this year, since the respiratory tract is one of the main routes for viral infection. It was not until 2025 that the development of lung organoids from bats of the *Rousettus leschenaultia* species was reported. These organoids successfully mimic the structure and morphology of the pulmonary epithelium and express human-like coronavirus entry receptors — ACE2 receptors (angiotensin-converting enzyme 2) and TMPRSS2 (transmembrane protease serine group 2). This model is very much in demand and represents a great opportunity to study infections originating from bats [59].

According to several researchers, integrating organoid cultures into epidemiological forecasting contributes to addressing questions regarding virus cross-species transmission, especially after substantial optimization of human organoid systems [24, 60, 61]. As an example of an optimized system, D. Holthaus et al. imply a harmonized cross-species organoid culture system for animal infectious disease modeling [62]. To this end, intestinal organoids derived from stem cells of four species (human, mouse, pig and chicken), which are important hosts of *Apicomplexa toxoplasma* and other protozoa as agents of zoonotic infections, were developed using the Transwell platform [62]. In this aspect, the study devoted to the cross-species analysis of the transcriptome of cells of the ileum epithelium of the mouse, bat, pig, macaque and human is also of interest,

providing information on the cellular composition of these organs and their functional purpose in 4 mammalian and human species. The results also showed that bats and humans have similar gene expression patterns, which is important for studying drug metabolism. In all likelihood, these data are also important for the design of cross-species organoids [63].

The lack of animal models is due to individual animal diversity and other problems (especially in capturing and surveying wildlife). In this aspect, organoid systems represent an excellent substitute for studying cross-species and species-specific infectivity of viruses. The maintenance and differentiation of organoids from different domestic and wild animal species requires species-specific optimization of culturing conditions. For example, the generation of mouse intestinal organoids requires conditioned media containing appropriate stem cell growth and differentiation factors, which do not yet exist for most animal species [64–66]. The problem of the difficulty of experimentally confirming susceptibility to coronavirus in most animal species, especially wild animals, has been highlighted by other researchers. They also believe that substantial optimization of human organoids will help in solving the issues of epidemiological prediction [24, 44, 60].

After obtaining a certain organoid culture of animals, it is necessary to characterize it authentically, e.g. for cell heterogeneity and lineage differentiation (gene expression), etc., in order to achieve this. Various cell markers are required for this purpose. Such markers exist for humans and mice, but are very limited in most animal species [61, 67].

Despite this, research related to animal organoids has intensified in the last 10 years [59, 66, 68–70].

Animal farms where several different animal species are kept, especially when wild animals may be in the environment, are a possible site for the emergence of new virus strains and their transmission to humans. Farm animal organoids play an important role in the study of zoonotic and reproductive diseases, not only for the improvement of agricultural production but also for public health. Intestinal organoids derived from crypts or pluripotent stem cells can serve as models for investigating the mechanisms of intercellular or pathogen-host interactions in zoonotic infections of the gastrointestinal tract, in which animals can serve as asymptomatic carriers of the disease [69, 70].

Intestinal organoids of the main species of farm animals: domestic pig (*Sus scrofa*) [71, 72], cattle (*Bos taurus*) [68, 73], sheep (*Ovis aries*) [74] and other animals have been used for successful modeling of various viral infections in animals and studying pathogen-host interaction in the intestine.

Organoids of the large and small intestine of marmosets (model nonhuman primates susceptible to gastrointestinal diseases) capable of passivation and long-term cultivation were obtained [75].

Organoid models reproducing various organs of domestic carnivores: cats (*Felis catus*) [76, 77], dogs (*Canis lupus*) [76-78] have also been obtained, since it cannot be excluded that domestic animals can be intermediate hosts in the transmission of viral infections [28, 68]. Various animal species, including domestic animals (cats, dogs, hamsters) and wild animals (lions, tigers), have been found to be infected with SARS-CoV-2 [79–81]. A large number of works are devoted to the use of animal organoids to study the pathogenesis of coronavirus infection. For example, infection of enteroids obtained from different segments of pig intestines with two types of coronavirus (*Porcine epidemic diarrhea virus* and *Transmissible gastroenteritis suum virus*) revealed the tropism of coronavirus to certain cells [71, 72]. Intestinal organoids (enteroids) of Chinese horseshoe bat (*Rhinolophus sinicus*) that reproduce intestinal epithelium and are susceptible to SARS-CoV-2 infection were obtained, in contrast to unsuccessful attempts using cell cultures [45]. Based on the results of *in silico* modeling of the molecular structure of the ACE2 receptor, the Malayan pangolin (*Manis javanica*) was the main candidate for the role of an intermediate host [82]. As noted above, organoids of bat lungs whose cells expressed ACE2 and TMPRSS2 entry receptors for coronavirus have been registered [59].

Although most studies with farm animal organoids are aimed at modeling infections, most authors agree that this cell technology holds great promise for applications in veterinary medicine, agriculture, biomedical sciences, and for assessing and predicting the ability of viruses to overcome the cross-species barrier.

Conclusion

In the practice of epidemiological surveillance of viral infections, in addition to modern molecular genetic technologies (PCR and sequencing), which are the main tools of epidemiological studies, the use of organoid (3D-cell) cultures is very relevant and promising.

The review analyzes numerous examples of the use of organoid (3D-cell) cultures of human and animal origin in modeling and studying the pathogenesis of infections caused by influenza, Zika, measles and other viruses. Special attention is given to the analysis of studies using such cultures in deciphering the pandemic of a new coronavirus infection, which made it possible to reveal the source and causes of its rapid spread around the world. The development of cross-species organoid cultures based on human and animal cells (wild and domestic) is of considerable interest in the study of the ability of viruses to overcome the cross-species barrier and adapt to the human body. Such information is necessary to build strategies to prevent and control cross-species transmission and to develop science-based interventions to prevent outbreaks. Despite intensive research, there are a number of limitations and challenges to cross-species organoid cultures. These in-

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clude several design features, issues of increasing their reproducibility, species-specific optimization and standardization of culturing protocols. The question of the possibility to construct organoids by fusion of human and animal cells remains open.

Thus, organoid (3D-cell cultures) of human and animal origin represent an effective model for studying the pathogenesis of viral infections, virus-host interactions, and for solving issues related to cross-species transmission of viruses, hence, for realizing the goals and objectives of epidemiological surveillance of viral infections.

Being widely implemented in virology and microbiology laboratories, these models will contribute to the development of science-based prediction of pathogen introduction from wild and farm animals into the human population, preventive measures, effective chemoprevention and treatment strategies for patients.

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ЮБИЛЕИ

К 60-летию академика РАН Василия Геннадьевича Акимкина

3 июля 2025 года отмечает 60-летие Василий Геннадьевич Акимкин, доктор медицинских наук, профессор, академик Российской академии наук, директор Центрального научно-исследовательского института эпидемиологии Роспотребнадзора.

Более 30 лет своей жизни Василий Геннадьевич посвятил проблемам эпидемиологии и организации эпидемиологического надзора за инфекционными болезнями, в том числе на основе молекулярно-генетических методов и цифровых технологий, профилактики инфекций, связанных с оказанием медицинской помощи, иммунопрофилактики, устойчивости микроорганизмов к противомикробным препаратам. За годы научной и научно-организационной деятельности В.Г. Акимкин создал научную эпидемиологическую школу и внёс неоценимый вклад в развитие отечественной эпидемиологической науки.

Василий Геннадьевич Акимкин окончил Томский медицинский институт (1988) и Военно-медицинскую академию (1994). В 2007–2010 гг. занимал пост Главного государственного санитарного врача Министерства обороны Российской Федерации. За годы службы им были разработаны и внедрены в практику деятельности Вооруженных Сил РФ научные принципы защиты военнослужащих и мирного населения от современных эпидемиологических угроз. В результате проведённых концептуальных реформ и санитарно-эпидемиологических мероприятий в армии достигнуто существенное снижение уровня заболеваемости военнослужащих вирусным гепатитом А, брюшным тифом, гриппом, внебольничными пневмониями и ветряной оспой.

Научная деятельность академика В.Г. Акимкина охватывает широкий круг задач современной медицины и здравоохранения. Им научно обоснованы, разработаны и внедрены в практику эпидемиологически безопасная система обращения с медицинскими отходами в России, современные методы и инновационные отечественные технологии очистки и дезинфекции воздуха в медицинских организациях. На основании фундаментальных научных исследований В.Г. Акимкиным разработано современное научное направление комплексного использования бактериофагов для лечения и профилактики инфекций, связанных с оказанием медицинской помощи, позволяющее в короткие сроки локализовать и ликвидировать длительно существующие эпидемические очаги. Изучена иммунологическая и эпидемиологическая эффективность вакцинации против гепатита В медицинского персонала и отдельных категорий пациентов с применением отечественных вакцин.

В 2018 г. Василий Геннадьевич Акимкин принял руководство Центральным научно-исследовательским институтом эпидемиологии Роспотребнадзора, и сегодня его имя неразрывно связано с новейшей историей института. За это время ФБУН ЦНИИ Эпидемиологии Роспотребнадзора стал крупнейшим развивающимся научно-производственным комплексом, решающим задачи в области эпидемиологического надзора, диагностики, профилактики и лечения широкого спектра инфекционной и неинфекционной патологии человека. Благодаря организаторской деятельности В.Г. Акимкина был введён в эксплуатацию Научный центр по профилактике и борьбе со СПИДом; ФБУН ЦНИИ Эпидемиологии Роспотребнадзора стал девятым в мире международным референс-центром Продовольственной и сельскохозяйственной организации Организации Объединённых Наций по устойчивости к противомикробным препаратам.

В 2020 г., в период начала пандемии новой коронавирусной инфекции, под руководством В.Г. Акимкина в короткие сроки были организованы действия сотрудников ФБУН ЦНИИ Эпидемиологии Роспотребнадзора по созданию, регистрации и массовому промышленному выпуску уникальных тест-систем (наборов реагентов) для диагностики COVID-19. Было выполнено 8 распоряжений

Правительства Российской Федерации по обеспечению отечественными тест-системами медицинских учреждений России и 40 стран. Проведено рекордное количество исследований (более 5 млн) методом ПЦР-диагностики населения г. Москвы и Московской области, выполнено более 1 млн серологических тестов на наличие антител против новой коронавирусной инфекции у населения, проведено секвенирование более 300 тыс. штаммов SARS-CoV-2.

Под руководством В.Г. Акимкина развёрнута беспрецедентная деятельность по созданию базы данных геномов возбудителей инфекционных болезней (Российская платформа агрегации геномов возбудителей инфекционных болезней — VGARus), включающей на сегодня более 400 тыс. образцов геномов более 80 различных патогенов. Сегодня в Институте продолжается работа по расширению спектра изучения геномов возбудителей других инфекционных болезней, разрабатываются и внедряются в практику инновационные решения на основе технологии CRISPR-Cas и метода петлевой изотермической амплификации нуклеиновых кислот, позволяющие проводить диагностику инфекционных болезней в 3–4 раза быстрее, чем стандартным методом ПЦР.

Академиком РАН В.Г. Акимкиным впервые в Российской Федерации разработаны научные и методологические основы функционирования геномного эпидемиологического надзора, основанного на мониторинге генетических свойств возбудителей инфекционных болезней — ведущего («движущего») фактора развития эпидемического процесса.

В.Г. Акимкин возглавляет Президиум Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов; удостоен золотой медали им. В.И. Покровского — высшей награды Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов и Евро-Азиатского общества специалистов по инфекционным болезням.

В.Г. Акимкин — автор более 1500 опубликованных научных работ (в том числе 20 монографий, 14 руководств для врачей), 79 патентов РФ на изобретения, 46 баз данных, 111 программных продуктов для ЭВМ, в том числе 8 международных публикаций по системе PCT, более 40 методических рекомендаций и пособий для врачей. Индекс Хирша — 33, Web of Science — 14, SCOPUS — 19.

Под его руководством защищено более 40 кандидатских и докторских диссертаций.

Василий Геннадьевич Акимкин ведет активную общественную и научную деятельность: он является председателем Президиума Всероссийского научно-практического общества эпидемиологов, микробиологов и паразитологов; членом Бюро Секции профилактической медицины Отделения медицинских наук РАН; председателем Комиссии Научного совета по микробиологии, эпидемиологии и инфекционным болезням Отделения медицинских наук РАН; членом двух диссертационных советов по специальности «Эпидемиология» на базе ФБУН ЦНИИ Эпидемиологии Роспотребнадзора и ФГБУ НИЦЭМ им. Н.Ф. Гамалеи; учёным секретарем Экспертного совета ВАК РФ по медико-профилактическим наукам; членом Бюро Комиссии по государственному санитарно-эпидемиологическому нормированию Роспотребнадзора; входит в редакционные советы и редакционные коллегии более 20 научных журналов.

В.Г. Акимкин — заслуженный врач Российской Федерации (2010); имеет государственные награды: орден Почета (2006), орден Пирогова (2022). Является лауреатом правительственных и национальных премий: «Лучший врач года» в номинации «Санитарный врач» (2005), национальной премии лучшим врачам России «Призвание» в номинации «За вклад в развитие медицины, внесённый представителями фундаментальной науки и немедицинских профессий» (2011), премии Правительства в области науки и техники (2017), национальной премии лучшим врачам России «Призвание» в номинации «За вклад в развитие медицины, медицинской науки и здравоохранения, внесённый представителями науки — научными работниками и (или) врачами любых специальностей и (или) специалистами с высшим немедицинским образованием» (2023).

В.Г. Акимкин награждён медалями, почётными грамотами и имеет благодарности от президента Российской академии наук; министра обороны Российской Федерации; руководителя Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека; министра здравоохранения Российской Федерации; Аппарата Совета Федерации Федерального Собрания Российской Федерации; Аппарата Государственной Думы Федерального Собрания Российской Федерации, прокуратуры, Центральной избирательной комиссии и др.

Научная деятельность академика РАН, доктора медицинских наук, профессора Василия Геннадьевича Акимкина вносит значительный вклад в развитие отечественной науки.

Коллектив Центрального научно-исследовательского института Роспотребнадзора, многотысячный коллектив членов Общероссийской общественной организации «Всероссийское научно-практическое общество эпидемиологов, микробиологов и паразитологов», авторы и читатели научно-практических журналов «Вопросы вирусологии» и «Журнал микробиологии, эпидемиологии и иммунобиологии», учредителем и издателем которых является ФБУН ЦНИИ Эпидемиологии Роспотребнадзора, поздравляют Василия Геннадьевича Акимкина с 60-летним юбилеем!

Примите искренние пожелания процветания Вашему великому делу! От всей души желаем Вам здоровья, творческих сил и вдохновения для новых побед и великих свершений!

К 100-летию профессора Натальи Николаевны Костюковой

6 июля 2025 года отмечает свой 100-летний юбилей заслуженный деятель науки Российской Федерации, доктор медицинских наук, профессор Наталья Николаевна Костюкова, старейший сотрудник Национального исследовательского центра эпидемиологии и микробиологии имени почетного академика Н.Ф. Гамалеи.

Наталья Николаевна — высококвалифицированный специалист в области медицинской микробиологии и эпидемиологии, чей трудовой стаж составляет 76 лет.

Закончив с отличием 1-й Московский медицинский институт, Наталья Николаевна избрала путь учёного и посвятила свою жизнь служению медицинской науке. В 1947 г. поступила в аспирантуру Московского Научно-исследовательского института вакцин и сывороток имени И.И. Мечникова по специальности «микробиология», где выполнила и защитила кандидатскую диссертацию «Микрофлора гематогенных остеомиелитов». Далее вся её трудовая деятельность связана с НИЦЭМ им. Н.Ф. Гамалеи, в котором она трудится вот уже более 60 лет и вносит несомненный вклад в развитие отечественной медицинской науки.

Являясь с 1976 по 1992 г. руководителем лаборатории этиологии и эпидемиологии острых менингитов НИИЭМ им. Н.Ф. Гамалеи АМН СССР (позже — РАМН), Наталья Николаевна внесла существенный вклад в расшифровку этиологической структуры острых гнойных менингитов у детей, в том числе новорождённых, в результате чего были значительно расширены представления о возбудителях этих заболеваний в нашей стране.

В зоне строительства Байкало-Амурской железнодорожной магистрали (БАМ) ею были проведены широкомасштабные исследования по выявлению менингококковой инфекции, практическим результатом которых явилась осуществлённая под её руководством впервые в СССР вакцинация детей, предотвратившая развитие надвигавшейся эпидемии менингококковой инфекции в зоне строительства БАМ и в Республике Тува.

Наталья Николаевна является автором таких научных работ, как «Уроки дифтерии» (1999), «Эпидемический процесс гонококковой инфекции — анализ и современные тенденции» (2012), обзоров о современных менингококковых (2024) и пневмококковых (2023) вакцинах, дифтерийном бактерионосительстве (2018).

Н.Н. Костюкова — ведущий научный сотрудник НИЦЭМ им. Н.Ф. Гамалеи. Ей принадлежат свыше 250 научных трудов, в том числе монографии по частной медицинской микробиологии и

этиологической диагностике инфекций (2010) и оппортунистическим инфекциям: возбудителям и этиологической диагностике (2013). Под её руководством защищены докторская и 19 кандидатских диссертаций.

Наталья Николаевна много сил и внимания уделяет воспитанию научных кадров, в том числе консультируя аспирантов и докторантов. Её лекции, доклады всегда подготовлены на высоком научном уровне, чётко структурированы, содержат яркие, убедительные примеры, факты, обоснования, документы и научные доказательства, что способствует формированию у молодых учёных современного научного мышления.

В течение нескольких последних десятилетий она остается бессменным учёным секретарем Научного совета по комплексной проблеме «Микробиология».

Широкий кругозор и высокая эрудиция в сочетании с огромной творческой активностью и трудолюбием позволяют Наталье Николаевне Костюковой быть признанным научным лидером не только в нашей стране, но и за рубежом.

Распоряжением Президента Российской Федерации от 26.06.2025 № 251-рп «О поощрении» за заслуги в области здравоохранения и многолетнюю добросовестную работу заслуженному деятелю науки Российской Федерации, ведущему научному сотруднику, доктору медицинских наук, профессору Н.Н. Костюковой объявлена благодарность Президента Российской Федерации.

Коллектив НИИЭМ им. Н.Ф. Гамалеи, члены Общероссийской общественной организации «Всероссийское научно-практическое общество эпидемиологов, микробиологов и паразитологов», редакция «Журнала микробиологии, эпидемиологии и иммунобиологии» поздравляют Наталью Николаевну с юбилеем и желает ей мира и здоровья.

НЕКРОЛОГ

Памяти Виталия Александровича Романова

29 июня 2025 года на 84-м году жизни скончался Виталий Александрович Романов, заведующий кафедрой микробиологии с вирусологией и иммунологией, профессор, доктор медицинских наук, Почётный работник высшего профессионального образования России, Почётный профессор Ярославского государственного медицинского университета.

Вся трудовая научно-педагогическая деятельность Виталия Александровича Романова связана с Ярославским государственным медицинским университетом. После окончания аспирантуры на кафедре микробиологии Воронежского медицинского института в 1967 году он прошёл путь от ассистента до заведующего кафедрой микробиологии с вирусологией и иммунологией Ярославского государственного медицинского университета (с 1975 года).

Высококвалифицированный врач, учёный, педагог Виталий Александрович Романов оказывал большую помощь практическому здравоохранению в подготовке врачей-бактериологов, их сертификации и аттестации. Совместно с органами здравоохранения Ярославской области и Роспотребнадзором Виталий Александрович неоднократно принимал участие в организации и проведении противоэпидемических мероприятий в периоды проведения в нашей стране Олимпийских игр, Игр доброй воли, других массовых мероприятий, участвовал в расследовании причин вспышек различных инфекций и купировании их распространения.

Профессор В.А. Романов проявил себя выдающимся научным работником и педагогом, готовил дипломированных специалистов высшей квалификации в области микробиологии и иммунологии. Им подготовлены 26 кандидата наук и 2 доктора наук. В.А. Романов является автором 385 научных работ.

Награждён дипломом мэрии г. Ярославля за многолетний добросовестный труд, активное участие в общественной жизни и большой вклад в социально-экономическое развитие города, почётным знаком губернатора Ярославской области «За заслуги в науке», золотой медалью Вернадского Российской Академии Естествознания «За заслуги в науке», присвоены звания Почетный работник высшего профессионального образования РФ, Отличник здравоохранения РФ.

Коллектив Ярославского государственного медицинского университета и редакция журнала выражают глубокие соболезнования родным и близким Виталия Александровича.