



ORIGINAL STUDY ARTICLE

DOI: <https://doi.org/10.36233/0507-4088-224>

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Phylogenetic analysis of variants of the Puumala virus (Hantaviridae: *Orthohantavirus*) circulating in the Saratov region

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Abstract

The objective is to determine the complete nucleotide sequence and conduct a phylogenetic analysis of genome variants of the Puumala virus isolated in the Saratov region.

Materials and methods. The samples for the study were field material collected in the Gagarinsky (formerly Saratovsky), Engelsky, Novoburassky and Khvalynsky districts of the Saratov region in the period from 2019 to 2022. To specifically enrich the Puumala virus genome in the samples, were used PCR and developed a specific primer panel. Next, the resulting PCR products were sequenced and the fragments were assembled into one sequence for each segment of the virus genome. To construct phylogenetic trees, the maximum parsimony algorithm was used.

Results. Genetic variants of the Puumala virus isolated in the Saratov region have a high degree of genome similarity to each other, which indicates their unity of origin. According to phylogenetic analysis, they all form a separate branch in the cluster formed by hantaviruses from other subjects of the Volga Federal District. The virus variants from the Republics of Udmurtia and Tatarstan, as well as from the Samara and Ulyanovsk regions, are closest to the samples from the Saratov region.

Conclusion. The data obtained show the presence of a pronounced territorial confinement of strains to certain regions or areas that are the natural biotopes of their carriers. This makes it possible to fairly accurately determine the territory of possible infection of patients and/or the circulation of carriers of these virus variants based on the sequence of individual segments of their genome.

Keywords: *hantaviruses; Puumala orthohantavirus; nucleotide sequence; Saratov region; Phylogeography*

For citation: Krasnov Ya.M., Naidenova E.V., Guseva N.P., Polunina T.A., Sharapova N.A., Sosedova E.A., Kotova N.V., Zakharov K.S., Kazantsev A.V., Domanina I.V., Chekashov V.N., Shilov M.M., Kondratiev E.N., Osina N.A., Kutyrev V.V. Phylogenetic analysis of the genome variants of the Puumala virus (Hantaviridae: *Orthohantavirus*) isolated in the Saratov region. *Problems of Virology (Voprosy Virusologii)*. 2024; 69(2): 162–174. DOI: <https://doi.org/10.36233/0507-4088-224> EDN: <https://elibrary.ru/ijmubu>

Funding. This study was not supported by any external sources of funding.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus author guidelines for animal use» (IAVES 23 July 2010). The study was approved at the meeting of the Bioethics Commission of the Russian Anti-Plague Institute «Microbe» (protocol No. 8, dated 21.11.2023).

ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ

DOI: <https://doi.org/10.36233/0507-4088-224>

Филогенетический анализ вариантов вируса Пуумала (Hantaviridae: *Orthohantavirus*), циркулирующих на территории Саратовской области

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Резюме

Цель работы – определение полной нуклеотидной последовательности и проведение филогенетического анализа вариантов геномов вируса Пуумала, выделенных на территории Саратовской области.

Материалы и методы. Образцами для исследования послужил полевой материал, собранный в Гагаринском (бывшем Саратовском), Энгельском, Новобураском и Хвалынском районах Саратовской области в период с 2019 по 2022 г. Для специфического обогащения генома вируса Пуумала в образцах использовали ПЦР и панель праймеров, подготовленную для данного исследования. Далее проводили секвенирование полученных продуктов реакции и сборку фрагментов в одну последовательность для каждого из сегментов генома вируса. При построении филогенетических деревьев применяли алгоритм maximum parsimony.

Результаты. Показано, что генетические варианты вируса Пуумала, выделенные в Саратовской области, имеют высокую степень подобия генома, что говорит о единстве их происхождения. По данным филогенетического анализа, все выделенные варианты вируса (за исключением изолятов вируса из Хвалынского района) образуют обособленную ветвь в кластере, сформированном хантавирусами из других субъектов Приволжского федерального округа. Самыми близкими к образцам из Саратовской области являются варианты вируса из республик Удмуртия и Татарстан, а также из Самарской и Ульяновской областей.

Заключение. Полученные данные указывают на наличие выраженной территориальной приуроченности штаммов к определенным регионам или областям, являющимся природными биотопами их носителей. Этот факт позволяет довольно точно определять территорию возможного инфицирования заболевших и/или циркуляцию переносчиков данных вариантов вируса по последовательности отдельных сегментов их генома.

Ключевые слова: хантавирусы; вирус Пуумала; нуклеотидная последовательность; Саратовская область; филогеография

Для цитирования: Краснов Я.М., Найденова Е.В., Гусева Н.П., Полунина Т.А., Шарапова Н.А., Соседова Е.А., Котова Н.В., Захаров К.С., Казанцев А.В., Доманина И.В., Чекашов В.Н., Шилов М.М., Кондратьев Е.Н., Осина Н.А., Кутырев В.В. Филогенетический анализ вариантов генома вируса Пуумала (Hantaviridae: *Orthohantavirus*), выделенных на территории Саратовской области. *Вопросы вирусологии*. 2024; 69(2): 162–174. DOI: <https://doi.org/10.36233/0507-4088-224> EDN: <https://elibrary.ru/ijmubu>

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

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

Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus author guidelines for animal use» (IAVES 23 July 2010). Исследование одобрено комиссией по биоэтике ФКУН «Российский противочумный институт «Микроб» Роспотребнадзора (протокол № 8 от 21.11.2023).

Introduction

It is known that the Volga Federal District (VFD) of the Russian Federation accounts for more than 80% of all annually registered cases of hemorrhagic fever with renal syndrome (HFRS) [1, 2], and the Saratov region is no exception. Thus, according to the data presented in the letter of the Chief State Sanitary Doctor of the Russian Federation A. Yu. Popova No. 02/4030-2024-32 dated 11.03.2024 «On the epidemiological situation of hemorrhagic fever with renal syndrome in 2023 and forecast for 2024», in 2023 in Russia 5093 cases of HPRS were registered

(3.47 per 100 thousand of the population; the average annual rate (2012–2023, excluding 2020 and 2021) was 5.34 per 100 thousand of the population), 84.7% of which were in the Volga Federal District.

Zoning of the territory of the Saratov region showed that in 16 out of 38 administrative districts for the 2010–2022 period, there was a high intensity of HFRS epidemic manifestations. Among the most unfavorable are the Gagarinsky district (formerly Saratovskiy district), as well as the Kumysnaya Polyana natural park, located in the city of Saratov. The area of the park is 4417 hectares [3]. As part of the organization of local government, the ter-

ritory of the Gagarinsky district from January 1, 2022 is included in the urban district of the city of Saratov, while not being part of the city limits.

In the European part of Russia, including the Saratov Region, the most common causative agent of HFRS is Puumala virus (*Puumala orthohantavirus*), the main natural host of which is the red vole (*Myodes glareolus* Schreber, 1780) [4, 5]. Puumala virus, like other hantaviruses, belongs to the *Orthohantavirus* genus of the *Hantaviridae* family [6]. The viral genome is represented by segmented single-stranded RNA of negative polarity. The large segment of the genome (L) encodes the viral RNA polymerase RdRp, the medium segment (M) encodes two surface glycoproteins G1 and G2, and the small segment (S) encodes the nucleocapsid protein (N) [7].

In a previous study [8], together with colleagues from the State Scientific Center of Virology and Biotechnology «Vector», we sequenced the complete genomes (segments S, M and L) of three samples taken from red voles caught in the Kumysnaya Polyana natural park (Saratov) in mid-2019. Analysis of the results obtained confirmed that it was Puumala virus that caused the outbreak of HFRS observed in Saratov at that time. The nucleotide sequences obtained were close to the previously described variants from the Republics of Tatarstan and Udmurtia, as well as the Samara region. There was conducted a comparative phylogenetic analysis of Puumala virus variants circulating in red voles captured in the Republic of Tatarstan [9, 10], and comparison with the samples from the Republic of Udmurtia and the Ulyanovsk Region [11]. The analysis showed the similarity of virus genomes from the studied regions with the variants isolated in Tatarstan. There was demonstrated that the identified Puumala virus isolates from the Tyumen, Arkhangelsk, and Omsk regions form a common Eastern Finnish branch, which raises additional questions about the distribution routes of the main carrier. Furthermore, data are presented comparing genic variants from Tatarstan, Udmurtia, Samara, and Saratov. The authors conclude that Puumala virus variants are likely to have arisen in these regions as a result of reassortment, since they contained segments S and L belonging to the Bashkir branch and segment M probably derived from sublineage ancestors from Kursk, Moscow and Ivanovo regions [12]. An attempt was also made to model the phylogeographic distribution of Puumala hantavirus throughout Europe during the last postglacial period [13].

Thus, the aim of the presented study is to determine the complete nucleotide sequence of the genome of various variants of Puumala virus isolated in the Saratov region and to conduct phylogenetic analysis of the obtained data.

Materials and methods

The samples for the study were organ samples of small mammals, mainly red vole, collected in 2019–2022 in the suburban and urban area of Saratov (Kumysnaya Polyana natural park), as well as in the Gagarinsky, Engelssky, Novoburassky and Khvalynsky districts of the Saratov region. The zoological group made regular visits to the

points determined by the annual monitoring plan, where the capture was carried out.

Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus author guidelines for animal use» (IAVES 23 July 2010). The study was approved by the Bioethics Commission of the Russian Anti-Plague Institute «Microbe» (protocol No. 8, dated 21.11.2023).

Nucleic acids were isolated using the AmpliPrime RIBO-prep RNA/DNA extraction kit, and reverse transcription was performed with the Reverta-L reagent kit (Central Research Institute of Epidemiology, Russian Federation). The presence of genetic material of hantaviruses was determined by RT-PCR with the OM-Screen-HPRS-RT reagent kit (Syntol Research and Development Company, Russian Federation).

A panel of 38 samples of small mammal lung suspensions in which Puumala virus RNA was detected by RT-PCR was compiled for the study. For specific enrichment of the viral genome (S, M, and L segments) in samples from field material, a set of primers calculated by the authors was used, which is listed in **Table 1**.

Further sequencing of the obtained PCR products was performed using a genetic analyzer (AB 3500xl and/or Ion S5), according to the recommendations of the manufacturer of this equipment (Thermo Fisher Scientific, USA). MEGA7 (<http://www.megasoftware.net/>) and UGENE (<http://ugene.net>) software were used to assemble sequenced fragments into a single sequence.

When constructing phylogenetic trees, the sequence of segment S (the central part of 1071 nucleotides), as well as most of the sequence of segments M and L of the Puumala virus genome, represented in the international database NCBI GeneBank, were used. This section of the work was performed using the maximum parsimony algorithm in the BioNumerics 7.6 software package (Applied Maths, Belgium).

Results and discussion

As a result of this study, nucleotide sequences of the genome of Puumala virus circulating in the Saratov region were obtained, 33 of which with the highest quality reads were deposited in the international database NCBI GenBank (**Table 2**).

A comparative analysis of 26 nucleotide sequences of the Puumala virus whole genome from suburban and urban areas of Saratov (Gagarinsky district) showed their high similarity with each other (98.6–100%), regardless of the year of isolation. Samples from Novoburassky (No. 272) and Engelssky (Nos. 99, 100, 101) districts have genome similarity of 98.4–99.1% with 26 samples from Gagarinsky district. Three samples from the Khvalynsky district, Saratov region (Nos. 117, 122, 129) have 95.5% similarity with all variants from the Gagarinsky district. Thus, a rather large number of differences among the variants of the virus genome from the Khvalynsky district was revealed compared to samples from the Engelssky, Gagarinsky and Novoburassky districts bordering each other (**Fig. 1**).

Using the available nucleotide sequences of the S segment of the Puumala virus genome presented in the inter-

Table 1. Oligonucleotide primer used in the work**Таблица 1.** Последовательности олигонуклеотидных праймеров, используемых в работе

Primer Праймер	Primer sequence Структура праймера	Annealing T, °C Температура отжига, °C	Amplicon length, bp Длина ампликона, п.н.
	Segment S Сегмент S		
Pum_S_1f	TAGTAGTAGACTCCTTGAAAAAGCTAC	50	370
Pum_S_1r	CAATGTCAATGGCGTTCAC		
Pum_S_2f	GAAGAATGGCAGATGCTGTGTCCC	58	454
Pum_S_2r	ACGGTCTGTCTTCCACGAGTTGAC		
Pum_S_3f	CGACTGGGATTGAACCTGATGATC	56	344
Pum_S_3r	CGGGTGTAAGTTCCCTCAGCTTTC		
Pum_S_4f	CATTTGAGGATAATTAATGGCTTTAGG	54	376
Pum_S_4r	AATCAACTTATCAATGTCTGCCAC		
Pum_S_5f	TGCGTAATATCATGAGTCCAGTGATG	56	355
Pum_S_5r	AGCCATCCCAGCAACATAAATG		
Pum_S_6f	ACATCGAATCTCCTAATGCACC	54	354
Pum_S_6r	TGATCTATGAGTGACTGAGCAAGG		
Pum_S_7f	TGCTCAGTCACTCATAGATCAGAAAG	53	400
Pum_S_7r	AGCTCAGTTTCACATTCTTGGG		
Pum_S_7-2f	AATCAGGAGCCCTTAAAGATATG	53	467
Pum_S_7-2r	TCAGCATGTTGAGGTAGTATGTTGTG		
Pum_S_fin_f	GTTTTGAATTAATGACTAATCAGGG	50	350
Pum_S_fin_r	TAGTAGTATGCTCCTTGAAAAAGCAATC		
	Segment M Сегмент M		
Pum_M_1f	TAGTAGTAGACTCCGCAAGAAGAAGC	54	386
Pum_M_1r	ATCCTCTCAAATTCACCTTCACTGC		
Pum_M_2f	CATGGGAAATTAAGGTGATCTTG	54	309
Pum_M_2r	AATTGCCCTGAAACACAGTATG		
Pum_M_3f	GTTTGATCCCTACTTTAGTGGTTG	53	349
Pum_M_3r	CTTCATACTATCACAGATGTCACC		
Pum_M_4f	ATACTGTGTTTCAGGGCAATTGG	56	386
Pum_M_4r	CCTGTACTTTCCAGCAATACGTAG		
Pum_M_5f	ATCGTTCTGCTGAAGTCTTTCAAG	56	345
Pum_M_5r	AAGACTGTGCATTGTGTGTCTTCTC		
Pum_M_6f	ACTGGGTTTCATCTCATTACCTGG	54	341
Pum_M_6r	TAACTCTACGGCAAGAGAGTGTG		
Pum_M_7f	GAGTTATGTGTACCAGGTCTTCACGG	55	340
Pum_M_7r	GCTTGAAGGGCAGATGTTGTTG		
Pum_M_8f	ATGGGCTCGATGGTCTGTGAGG	58	380
Pum_M_8r	TATCCCAGACCCGTGTGTGTGTC		
Pum_M_9f	CTCTCTTTAGGTATCGGAGTCGG	55	392
Pum_M_9r	CAGCAGTTTGCCAAGGATAAGC		
Pum_M_10f	AAACTGCATTTCAATGCTATGGTTC	55	326
Pum_M_10r	TTGAAATTGTCCCTATCAAACACAC		
Pum_M_10-2f	GGCTGTAACCCACTTGATTGCC	58	430
Pum_M_10-2r	CCATCAAACCTGGCACACAGGTGTTG		
Pum_M_11f	TAATATTTAAGCAATGGTGCACTAC	53	452
Pum_M_11r	CCTTAATTGAAGTAAGAAATGCAG		
Pum_M_12f	GATGGGAATACAATTCAGGATAC	53	307
Pum_M_12r	GCCTTAATTGAAGTAAGAAATGCAG		
Pum_M_13f	GAATGGATTGATCCTGACAGTTCAC	56	502
Pum_M_13r	ATCCAATTTCCATTGAGGACCC		

Продолжение табл. 1 см. на стр. 166.

Primer Праймер	Primer sequence Структура праймера	Annealing T, °C Температура отжига, °C	Amplicon length, bp Длина ампликона, п.н.
Pum_M_13-2f	CAATGTGTTACGGATCTACTACAGC	54	393
Pum_M_13-2r	GCAACTATCTACTAAGGCTTATGCTC		
Pum_M_fin_f	GACAGGATACAACCAAGCAGATAGTG	54	417
Pum_M_fin_r	TAGTAGTAGACTCCGCAAGAACAAAAG		
	Segment L Сегмент L		
Pum_L_1f	TAGTAGTAGACTCCGAGATAGAGAAG	51	468
Pum_L_1r	TGTGCTTCTACCTGTAGTTGTTGCTC		
Pum_L_2f	ATGATGTGATACAAAGCATGGAG	53	453
Pum_L_2r	TCCTAAAGCCAGATTGACAATTAC		
Pum_L_3f	GTTTATTGAGCAACAACACTACAGGTAG	53	394
Pum_L_3r	GCCCAGTTACTTCTTTAAATGC		
Pum_L_4f	TGTAAGAATTGGCTCGGAACCTGATC	56	421
Pum_L_4r	TCAATCAATGCCTTCGACTTAGGATC		
Pum_L_5f	ATCTCAAAGGATTCAAAGAAAGGG	54	391
Pum_L_5r	TCAAGTGATTTAGATGGCAGGATAC		
Pum_L_6f	CGTGATATTACTGAATCTCTTATTGC	53	406
Pum_L_6r	TGAGATGTGGAAAGAAAAGAAATGG		
Pum_L_7f	CTTCTTGCTACAGCTACATGGTTTC	54	410
Pum_L_7r	ACGATAATGCTTATACACGACCC		
Pum_L_8f	AAGACACTATTAGTTAGCTTAGCCC	52	354
Pum_L_8r	GCTGTTCAACAACACTACCTGATTG		
Pum_L_9f	TTCATCTTGAAACAGTTGAATGGG	54	438
Pum_L_9r	CTTTGGTATTTCTTAACAATTCTTGC		
Pum_L_9-2f	TCATCTTGAAACAGTTGAATGGG	54	341
Pum_L_9-2r	AAACGAATTGCCTCAATGAGAG		
Pum_L_10f	GTCAGGAGCATTACAAGAAGATGGTC	56	369
Pum_L_10r	CAACGAGGACTGGATTTCACTTTCTC		
Pum_L_11f	ATTCAACAGGCTTTAGAGAAGGC	54	450
Pum_L_11r	GATATAATTTGGCCACACACG		
Pum_L_12f	AATATGGGTGAATTGTCTGATGAAG	54	440
Pum_L_12r	GAATGTAGATAGAACTCCGCATTTG		
Pum_L_13f	GGCAGGAACTTTCATTGGCATG	57	440
Pum_L_13r	GACATAGAACCATCACCACCTAACGG		
Pum_L_14f	TTATATGGTACTGCTCCTGGTATGG	54	383
Pum_L_14r	AGGATGTTGTTCACTCCAAAGCTC		
Pum_L_15f	TTTGTAGGTAAAGTTCAGTGGAAAAG	53	459
Pum_L_15r	TTGGTCTATGAATTTGTCTTGTG		
Pum_L_15-2f	CTTTGGAGTGAACAACATCCTGTG	54	320
Pum_L_15-2r	ATCCCTCCAGGCATATCTTTAG		
Pum_L_16f	GGATATGGAGCTCTTTCAAACACTTG	55	387
Pum_L_16r	CACAATTACAATCCTCGACTTTCC		
Pum_L_17f	TTATTGGTTGAAGACTATGTCTCTTG	52	441
Pum_L_17r	ATTAAATGTIACCCTCAAATCTCC		
Pum_L_18f	CTTTATGAAGGAGATTTGAGGGTAAC	53	423
Pum_L_18r	CCTTAGATTATGGTATGCATGG		
Pum_L_19f	CTAATGGATTTAGGGCTATGGC	54	385
Pum_L_19r	TGACCATTGAGTACTAGAGATTGTGC		
Pum_L_20f	AGCACAATCTCTAGTACTCAATGGTC	53	405
Pum_L_20r	CGAACTCTGTAAATCATAACGGATC		
Pum_L_fin_f	GTCCAAGCATTACAATTTCCATAC	52	304
Pum_L_fin_r	TAGTAGTATGCTCCGAGAAAAGAGC		

Table 2. List of Puumala orthohantavirus sequences obtained in this study and deposited in the NCBI GenBank database

Таблица 2. Нуклеотидные последовательности вируса Пуумала, полученные в результате исследования

No. №	Sample Образец	Place of the sample collection Место получения образца	Year of collection Год сбора материала	GenBank Accession Number Номер в базе NCBI GenBank		
				segment S сегмент S	segment M сегмент M	segment L сегмент L
1.	114		2019	OL343591.1	OL343569.1	OL343547.1
2.	131		2019	OL343593.1	OL343571.1	OL343549.1
3.	296		2019	OL343594.1	OL343572.1	OL343550.1
4.	348		2019	OL343595.1	OL343573.1	OL343551.1
5.	420		2019	OL343596.1	OL343574.1	OL343552.1
6.	422		2019	OL343597.1	OL343575.1	OL343553.1
7.	439		2019	OL343598.1	OL343576.1	OL343554.1
8.	525		2019	OL343599.1	OL343577.1	OL343555.1
9.	645		2019	OL343600.1	OL343578.1	OL343556.1
10.	656		2019	OL343601.1	OL343579.1	OL343557.1
11.	696		2019	OL343602.1	OL343580.1	OL343558.1
12.	701		2019	OL343603.1	OL343581.1	OL343559.1
13.	836	Gagarinsky district	2019	OL343604.1	OL343582.1	OL343560.1
14.	988	Гагаринский район	2019	OL343605.1	OL343583.1	OL343561.1
15.	989		2019	OL343606.1	OL343584.1	OL343562.1
16.	1039		2019	OL343585.1	OL343563.1	OL343541.1
17.	1042		2019	OL343586.1	OL343564.1	OL343542.1
18.	1059		2019	OL343587.1	OL343565.1	OL343543.1
19.	1081		2019	OL343588.1	OL343566.1	OL343544.1
20.	1107		2019	OL343589.1	OL343567.1	OL343545.1
21.	1109		2019	OL343590.1	OL343568.1	OL343546.1
22.	1186		2019	OL343592.1	OL343570.1	OL343548.1
23.	3		2022	OQ032670.1	*	*
24.	29		2022	OQ032672.1	*	*
25.	30		2022	OQ032673.1	*	*
26.	31		2022	OQ032671.1	*	*
27.	99		2022	OQ032667.1	*	*
28.	100	Engelssky district	2022	OQ032669.1	*	*
29.	101	Энгельсский район	2022	OQ032668.1	*	*
30.	117		2021	OR999067	*	*
31.	122	Khvalynsky district	2021	OR999068	*	*
32.	129	Хвалынский район	2021	OR999069	*	*
33.	272	Novoburassky district	2021	OR999070	*	*
		Новобурасский район				

Note. * – Data at the analysis stage.

Примечание. * – данные на этапе анализа.

national genetic database, an analysis including 368 viral variants was performed (Fig. 2 a) and a general phylogenetic tree was constructed. Comparison of sequences of M or L segments of the hantavirus genome is limited by their much smaller number relative to the S segment represented in the database, but the clustering pattern changes insignificantly, retaining, as a rule, the same mutual arrangement.

When phylogenetic relatedness was established, the S segment of the Puumala virus genome variants from the Saratov region formed a common cluster with viral sequences from the Ulyanovsk, Samara, and Penza regions, as well as from the Republics of Tatarstan, Bashkortostan, and Udmurtia. Sequence variants of the same genome segment obtained in other Russian territories (Omsk, Tyumen, Arkhangelsk, Moscow, Ivanovo and

Kursk regions, and the Republic of Karelia) were significantly distant from the cluster formed by sequences from the above-mentioned subjects of the Volga Federal District. It is worth noting that there are two samples (NCBI, MT822195 and MT822196) on the same branch formed by genomes from Samara region, obtained from clinical material collected in January 2020 in Switzerland (Fig. 2 a, b). But the annotation of these sequences does not provide information about the possible site of infection of HPRS patients, making it difficult to draw clear conclusions. Otherwise, the cluster formed by viral sequences (segment S) from the territory of the Volga Federal District did not include any variants from neither the other Federal Districts of Russia, nor any nearby or far abroad countries.

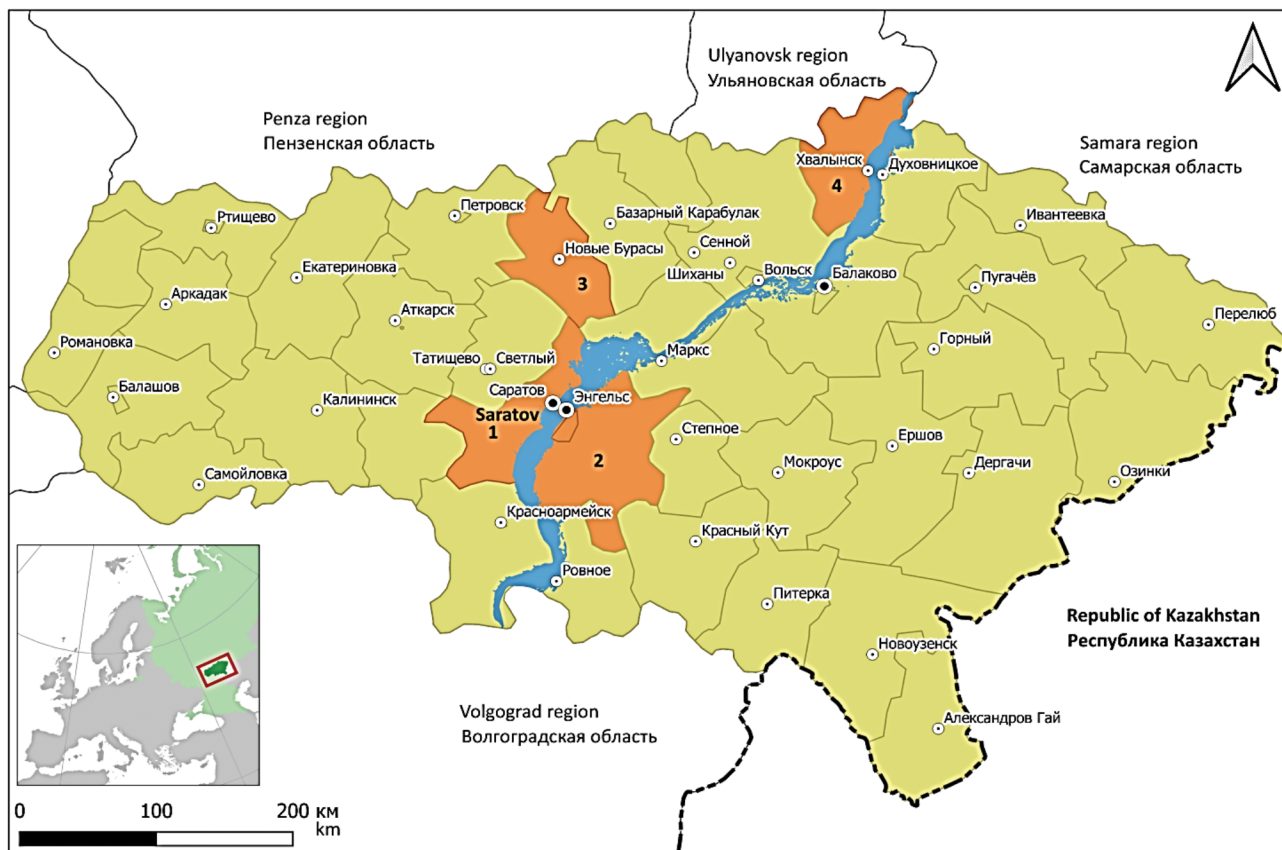


Fig. 1. Administrative division of the Saratov region.

The city district of Saratov (1), Engelssky (2), Novoburassky (3) and Khvalynsky (4) districts are highlighted in color.

Рис. 1. Административное деление Саратовской области.

Цветом выделены Гагаринский район и городской округ Саратова (1), Энгельсский (2), Новобурасский (3) и Хвалынский (4) районы.

Within cluster 1 (Fig. 2 *a, b*), formed by Puumala virus genomes from samples from the territory of the Volga Federal District, the degree of sequence similarity is 91.4–100%. Despite the fact that only the central part of the S segment sequence (1071 bp) was used in the comparative analysis, each variant from this cluster has at least 62 unique mutations that are common to all samples from cluster 1 and distinguish these variants from all others (Fig. 2 *a, b*). Figure 2 *b* shows that variants of the Puumala hantavirus genome from the Saratov urban district and Engelssky district, and one from Novoburassky district, form a compact group in the form of a separate branch. The places where carriers of the Puumala virus were captured from Udmurtia within the urban district of Saratov were separated from each other from several hundred meters to 9 km. Between the places of capture of Puumala virus carriers within the urban district of Saratov and Engelssky district, the distance in a straight line is 14–19 km, and for a sample from the Novoburassky district – about 46 km. Despite the significant remoteness, a high degree of similarity (98% or more) is observed for Puumala virus genome variants from these territories, which may indicate a single biotope of the main carriers and their free movement within these regions. The Khvalynsky district is located in the north of the Saratov region (more than 100 km distance of from the other re-

gions studied) and borders on the Ulyanovsk and Samara regions (Fig. 1). This fact is clearly reflected in the location of Puumala virus variants in cluster 1 (Fig 2 *b*). There was demonstrated that the samples obtained from Khvalynsky district and variants from Gagarinsky, Engelssky and Novoburassky districts are on the different branches differing from them by 55–78 single nucleotide polymorphisms (SNPs) and have similarity with the latter of 92.7–96.9%. The closest to hantavirus variants from the Khvalynsky district are the genomes of Puumala virus from the Samara region, followed by its variants from the Republic of Tatarstan and Ulyanovsk region (Fig. 2 *b*).

A detailed examination of the location of genome variants (S segment) of Puumala virus from Saratov region (Fig. 2 *b*) shows very close affinity between samples from Gagarinsky district, as well as their great similarity with samples from the Engelssky and Novoburassky districts. The difference between the sequence of the S segment (1071 bp) of the Puumala virus variant isolated in August 2021 in the Novoburassky district and the closest Puumala variant obtained in May 2019 in the territory of the Gagarinsky district is 5 SNPs. The differences between the sequences of the S segment (1071 bp) of the Puumala virus variant detected in July 2022 in the Engelssky district from the closest one obtained in February 2020 during the survey of the Gagarinsky district

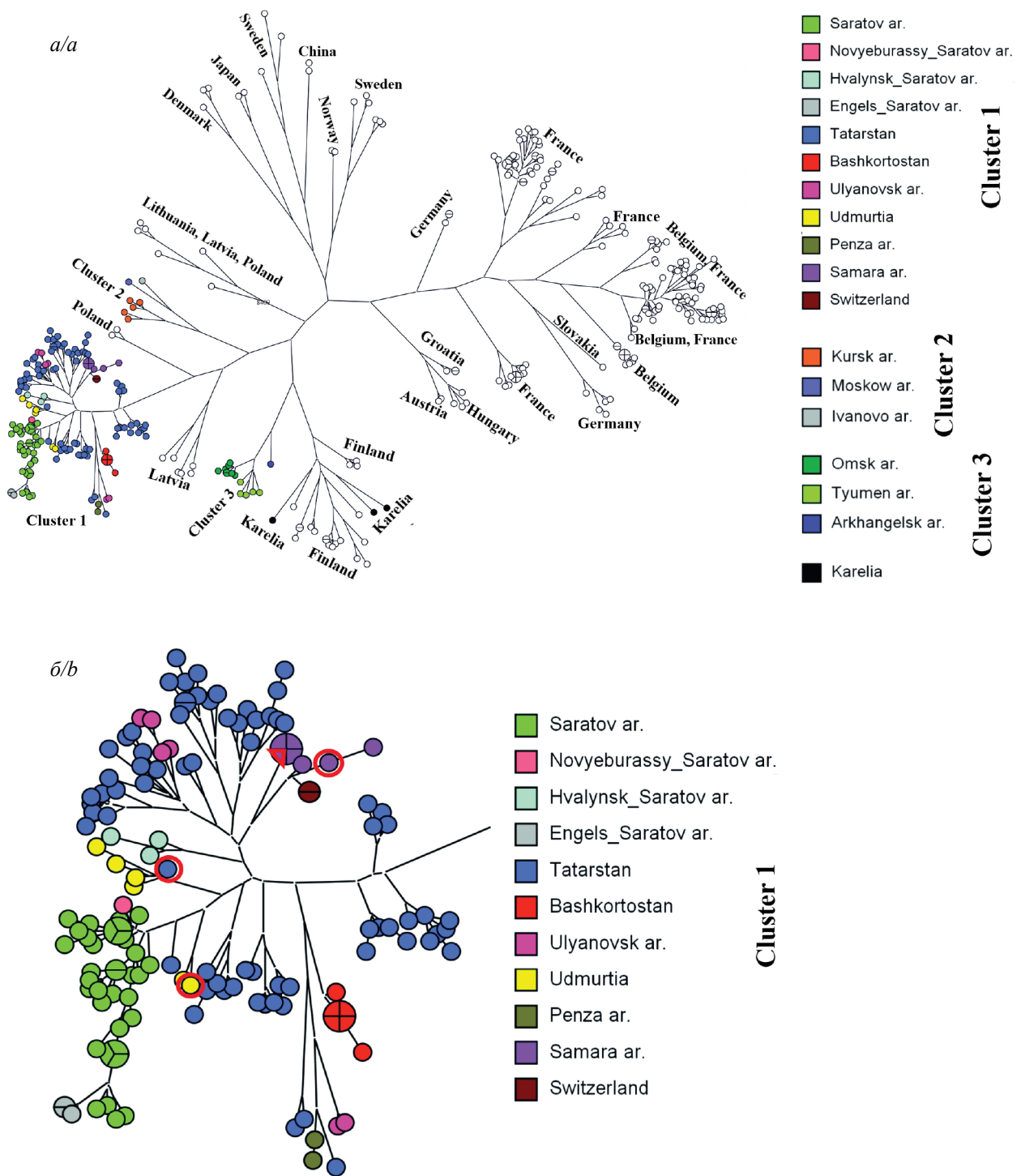


Fig. 2. Phylogenetic tree showing the close relationship between 368 genome variants (segment S) strains of the *Puumala orthohantavirus* from Russia, near and far abroad countries.

a – for three clusters; *b* – enlarged fragment of the phylogenetic tree – cluster 1 in Figure 2 *a*. The close relationship between genome variants (segment S) of 151 strains of the *Puumala orthohantavirus* from the Volga Federal District is shown. In Figure 2 *b*, samples circled in red remained the only variants from the republics of Bashkortostan and Tatarstan, Samara and Ulyanovsk regions in the case of phylogenetic trees for segments M and L.

Рис. 2. Филогенетическое дерево, показывающее близость родства между 368 вариантами генома (сегмент S) вируса Пуумала с территории России, стран ближнего и дальнего зарубежья.

a – по трем кластерам; *b* – увеличенный фрагмент филогенетического дерева – кластер 1 на рис. 2 *a*. На рис. 2 *b* образцы, обведенные красным цветом, остались единственными вариантами из республик Башкортостан и Татарстан, Самарской и Ульяновской областей в случае филогенетических деревьев по сегментам M и L.

are 15 SNPs. Between all investigated variants of Puumala virus circulating in the Gagarinsky district of Saratov region, the maximum difference in the sequence of the S segment (1071 bp) is 22 SNPs (98% similarity or more).

Molecular clock analysis performed by researchers [14, 15] showed that the S segment of the hantavirus genome evolves at a rate of about $6.7-10^{-4}$ single substitutions per year. These data suggest a rather slow change rate in the genome of Puumala virus in nature. In general, clustering by the S segment shows a common origin from one ancestral virus, or several close variants of it, for all samples from the territory of the Volga Federal District used in the analysis.

Comparative phylogenetic analysis of Puumala virus by M or L segments of samples obtained in the territory of the Saratov region shows certain changes in clustering, however, preserving the general trends of mutual arrangement.

It is worth noting that for segments M or L there is significantly less data on the genome sequences of the Puumala virus than for segment S. In Figure 2B, samples circled in red remained the only variants from the republics of Bashkortostan and Tatarstan, Samara and

Ulyanovsk regions in the case of phylogenetic trees for segments M and L.

Figure 3 shows the result of phylogenetic clustering between 94 genome variants (M segment, 2920 bp locus) of Puumala virus from the territory of Russia, near and far abroad. The phylogenetic tree obtained shows that the samples from the Gagarinsky district (Saratov ar.) still form a homogeneous group and are in the same cluster with the variants from the Volga Federal District, while the closest in similarity to them are the virus variants from the Republics of Udmurtia (OP561835, similarity of 94.9–95.6%), Tatarstan (Z84205, similarity of 94.6–95.3%) and Samara region (AB433850, similarity of 91.3–92.0% and AB433852, similarity of 90.6–91.3%). It should be noted that when clustering by M segment, the virus genome sequences obtained from Moscow, Kursk, and Ivanovo regions are closer to the variants from the territory of the Volga Federal District than the variants from the Republic of Bashkortostan, which is part of the Volga Federal District. In the M segment, variants from Bashkiria form a separate branch that is not included in clusters 1 and 2. In contrast, during phylogenetic clustering of the virus by S

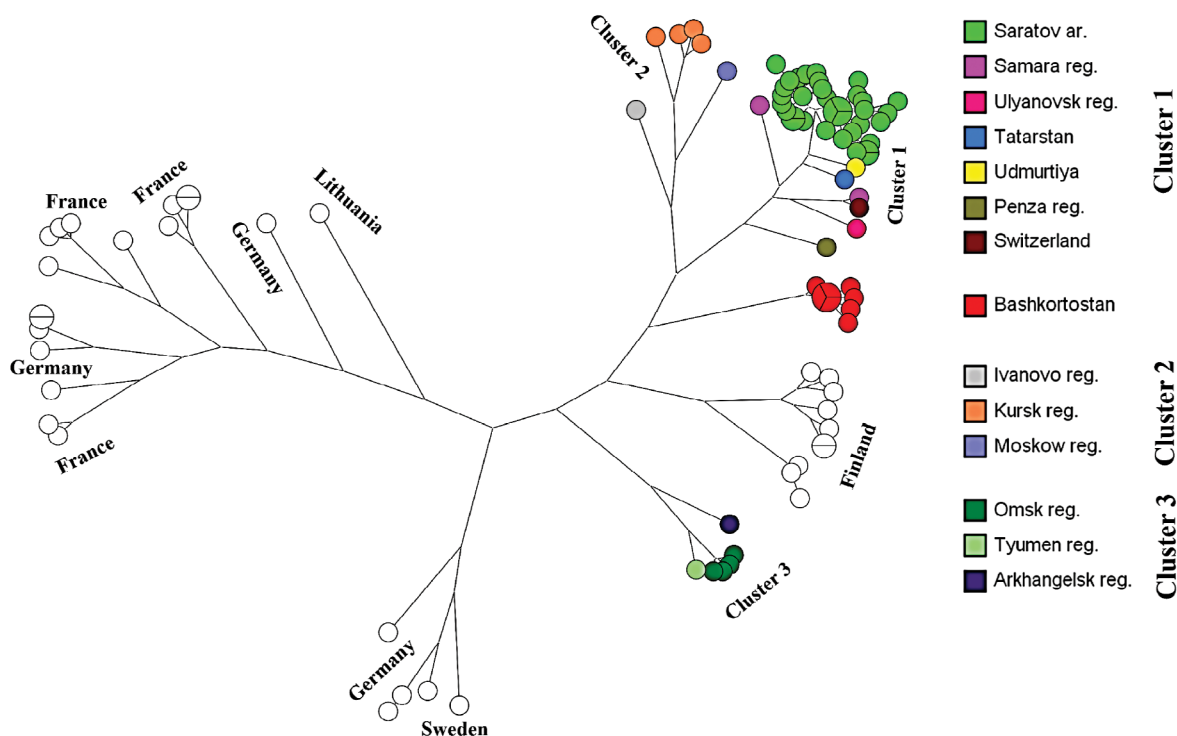


Fig. 3. Phylogenetic tree showing the close relationship between 94 genome variants (segment M) of Puumala orthohantavirus strains from Russia, near and far abroad countries.

Note: Bashkortostan (NCBI: AB297666, AF442614, KT885051, L08754, MH251332, MK496160, MZ673553, NC_077666); Kursk region (MZ580943, MZ580946, MZ580949, MZ580952); Ivanovo region (OP561838); Moscow region (OP561841); Samara region (AB433850, AB433852); Ulyanovsk region (OP561826); Tatarstan (Z84205); Udmurtia (OP561835); Penza region (OP561847); Switzerland (MT822194); Omsk region (AF367061, AF442615, AF442616, AF442617); Arkhangelsk region (OP561850); Tyumen region (OP561853).

Рис. 3. Филогенетическое дерево, показывающее близость родства между 94 вариантами генома (сегмент М) вируса Пуумала с территории России, стран ближнего и дальнего зарубежья.

Примечание: Башкортостан (AB297666, AF442614, KT885051, L08754, MH251332, MK496160, MZ673553, NC_077666); Курская обл. (MZ580943, MZ580946, MZ580949, MZ580952); Ивановская обл. (OP561838); Московская обл. (OP561841); Самарская обл. (AB433850, AB433852); Ульяновская обл. (OP561826); Татарстан (Z84205); Удмуртия (OP561835); Пензенская обл. (OP561847); Швейцария (MT822194); Омская обл. (AF367061, AF442615, AF442616, AF442617); Архангельская обл. (OP561850); Тюменская обл. (OP561853).

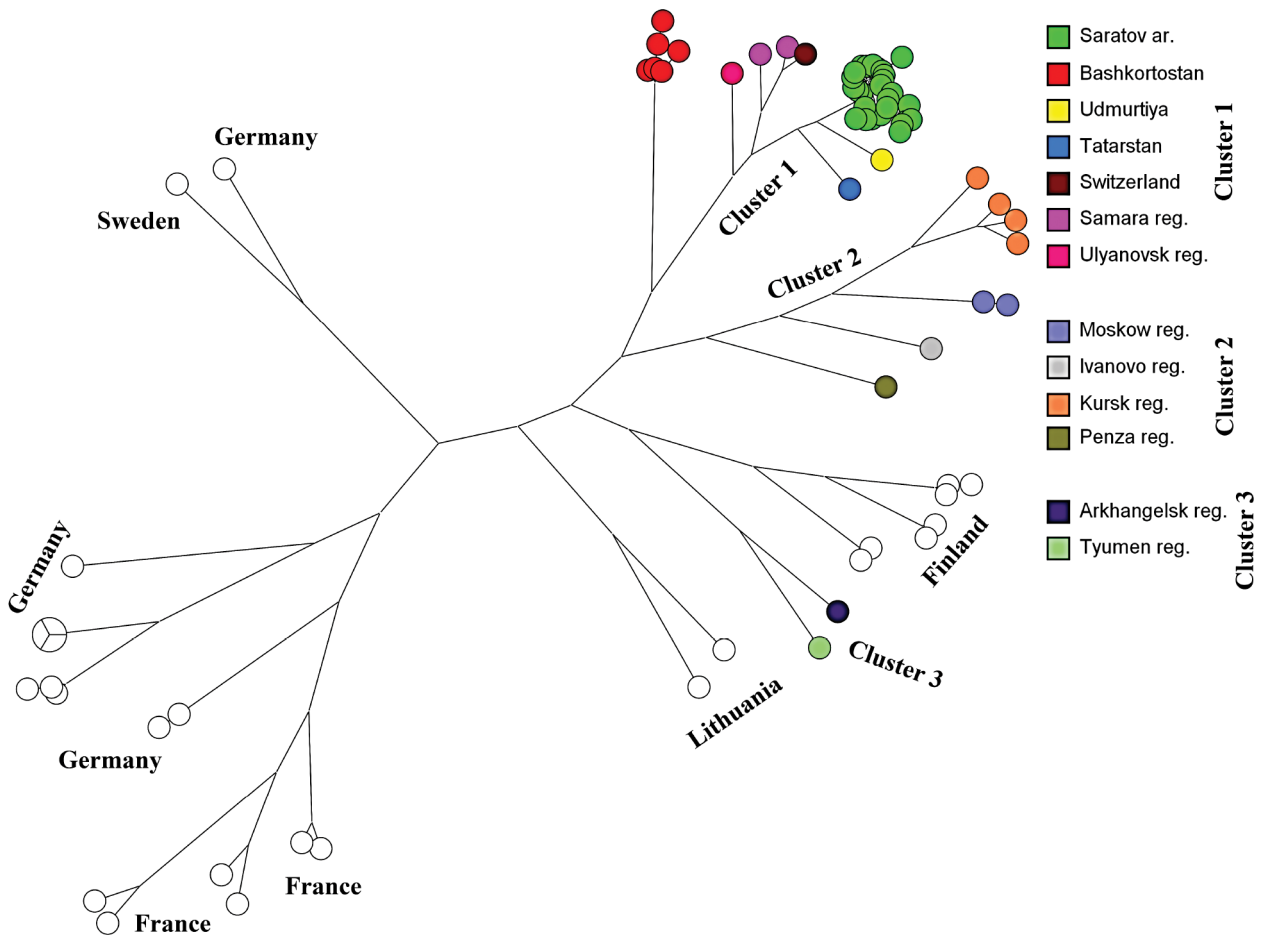


Fig. 4. Phylogenetic tree showing the close relationship between 76 genome variants (segment L) of *Puumala orthohantavirus* strains from the territory of Russia, near and far abroad countries.

Note: Bashkortostan (NCBI: AB297667, KT885050, MK496161, MK496164, MH251333, NC_077667); Samara region (AB574183, AB574184); Ulyanovsk region (OP561825); Udmurtia (OP561834); Tatarstan (EF405801); Швейцария (MT822193); курская обл. (MZ580944, MZ580947, MZ580953, MZ580950); Московская обл. (OP561840, OP561843); Иваново region (OP561837); Пенза region (OP561846); Архангельск region (OP561849); Тюмен region (OP561852).

Рис. 4. Филогенетическое дерево, показывающее близость родства между 76 вариантами генома (сегмент L) вируса Пуумала с территории России, стран ближнего и дальнего зарубежья.

Примечание: Башкортостан (AB297667, KT885050, MK496161, MK496164, MH251333, NC_077667); Самарская обл. (AB574183, AB574184); Ульяновская обл. (OP561825); Удмуртия (OP561834); Татарстан (EF405801); Швейцария (MT822193); курская обл. (MZ580944, MZ580947, MZ580953, MZ580950); Московская обл. (OP561840, OP561843); Ивановская обл. (OP561837); Пензенская обл. (OP561846); Архангельская обл. (OP561849); Тюменская обл. (OP561852).

segment, all sequences from the Volga Federal District were in the same cluster and significantly distant from variants from Central Russia. It is likely that the hantavirus obtained in the Republic of Bashkortostan had reassortment along the M segment with some variant of this virus from a region that is not part of the Volga Federal District.

Some authors' conclusion [12] that variants of Puumala virus likely arose in Tatarstan, Udmurtia, Samara, and Saratov as a result of reassortment, since they contained segments S and L belonging to the Bashkir branch and segment M derived from sublineage ancestors from Kursk, Moscow, and Ivanovo regions, was not confirmed in this study. Stable preservation of the unity of phylogenetic isolation of Puumala virus genomes on the territory of the subjects of the Volga Federal District is evident, with the exception of variants from the territory of Bashkortostan

for segment M (Fig. 3) and a sample from the Penza Region for segment L (Fig. 4). In Figure 3, which shows the M segment affinity, any of the variants of the Puumala virus genome in cluster 1 differs by at least 433 SNPs (similarity of 85.2%) from any of the variants of the Puumala virus genome in cluster 2. Variants from Bashkortostan differ by at least 531 SNPs (similarity of 81.8%) from any of the variants in both cluster 1 and cluster 2. The branch with the closest variants of Puumala virus from Finland has about 580 SNPs (similarity of 80.1%) from variants from Bashkortostan.

The lack of data from most regions of Russia on Puumala virus variants circulating in them makes it very difficult to draw conclusions about possible pathways of reassortant emergence.

Figure 4 shows the result of phylogenetic clustering between 76 genome variants (segment L, locus

at 6405 bp) of Puumala virus from Russia, near and far abroad. All variants from the Gagarinsky district (Saratov ar.) of the Saratov region are invariably in the same cluster with other Puumala viruses from the Volga Federal District. The closest to the variants from Saratov region, as in the case of segment M, are Puumala virus samples from the Republics of Udmurtia (OP561834, similarity of 95.2–95.8%) and Tatarstan (EF405801, similarity of 94.5–95.1%), as well as Samara region (AB574183 и AB574184, similarity of 91.7–92.7%). When analyzing segment L, variants of Puumala virus from the Republic of Bashkortostan, although located in the same cluster (cluster 1) together with variants of this virus from other subjects of the Volga Federal District, are significantly separated from them, maximum similarity of 86.1% (about 890 SNPs compared to the closest variant from the Ulyanovsk region in this cluster). The result of phylogenetic clustering of Puumala virus genome variants by segment L shows that the sample from Penza region is not located in cluster 1 together with virus variants from the Volga Federal District, but is in cluster 2 with variants from Ivanovo, Moscow, and Kursk regions. As can be seen from Figures 2B and 3, the sample from Penza region in terms of S and M segments belongs to cluster 1, which consists only of virus variants from the Volga Federal District. Thus, a possible reassortment of the Puumala virus variant from Penza region with variants of this virus from Ivanovo, Moscow, or Kursk regions, apparently as a result of migration of the red vole, is shown by the L segment. In previously published materials, the authors noted that this genome variant could have arisen as a result of two-step recombination [12].

Conclusion

The results of phylogenetic analysis of different variants of Puumala virus from Saratov region with the sequences presented in the NCBI GenBank database allow us to draw the following conclusions:

- all variants of Puumala virus strains circulating in the Saratov region have a high degree of similarity between genomes, which may indicate the unity of their origin;
- genetic sequences of Puumala virus from the Khvalynsky district have significant differences from variants obtained from the central districts of Saratov region.
- all genetic sequences of Puumala virus obtained from the urban district of Saratov, including those of genome segments S, M and L have no reassortment.

Summarizing the obtained data, we can note the presence of pronounced territorial confinement of Puumala virus strains (or rather their carriers) to certain regions or areas. This makes it possible to accurately determine the area of possible infection of patients and the habitat of carriers of these genetic variants of *Puumala orthohantavirus* by the sequence of viral genome segments.

The authors confirm that there is no conflict of financial/non-financial interests related to the writing of the article.

The material has been prepared for publication on the basis of the research performed within the framework of

R&D 92-2-21 Development of a system for verification of the results of indication and identification of pathogens of dangerous infectious diseases of bacterial and viral nature using molecular genetic methods (2021–2023).

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Received 05 February 2024

Accepted 02 April 2024

Published 27 April 2024

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Поступила 05.02.2024

Принята в печать 02.04.2024

Опубликована 27.04.2024