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Genetic features of the Puumala virus (Hantaviridae: *Orthohantavirus*) identified in the Moscow region

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Introduction. Puumala virus (family *Hantaviridae*, genus *Orthohantavirus*) is distributed in most regions of the European part of Russia. However, information about its genetic variants circulating on the territory of the Central Federal District is extremely scarce.
Materials and methods. Rodents' tissue samples were tested after reverse transcription by PCR for the presence of hantaviral RNA. The amplified fragments of the L segment were sequenced by the Sanger method. For two samples, sequences of all three segments were obtained using the NGS method. Phylogenetic trees were built in the MEGA-X software.
Results. Puumala virus was found in six samples. Based on the phylogenetic analysis of sequences of three segments, the obtained genetic variants belong to the sublineage previously designated as W-RUS.
Conclusion. A genetic variant of the Puumala virus, belonging to the subline W-RUS, circulates on the territory of the Volokolamsk district of Moscow region.

Keywords: *Puumala orthohantavirus; sublineage W-RUS; bank vole; phylogeography*

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Генетические особенности вируса Пуумала (Hantaviridae: *Orthohantavirus*), обнаруженного в Московской области

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Введение. Вирус Пуумала (семейство *Hantaviridae*, род *Orthohantavirus*) распространен в большинстве регионов Европейской части России. Однако сведения о его генетических вариантах, циркулирующих на территории Центрального федерального округа, крайне скудны.
Цель работы – изучение генетических вариантов вируса Пуумала, циркулирующих в грызунах на территории Волоколамского района Московской области.
Материалы и методы. Ткани грызунов исследовали методом ПЦР на наличие РНК хантавирусов. Амплифицированные фрагменты сегмента L секвенировали методом Сэнгера. Для двух образцов были получены последовательности всех трех сегментов методом NGS. Филогенетические деревья строили в программе MEGA X.
Результаты. В 6 исследуемых образцах был обнаружен вирус Пуумала. Филогенетический анализ, основанный на последовательностях трех сегментов, показал, что обнаруженные генетические варианты принадлежат к сублинии, обозначенной ранее как W-RUS.
Заключение. На территории Волоколамского района Московской области циркулирует генетический вариант вируса Пуумала, относящийся к сублинии W-RUS.

Ключевые слова: *ортохантавирус Пуумала; сублиния W-RUS; рыжая полевка; филогеография*

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Introduction

Representatives of the *Orthohantavirus* genus are among the most important zoonotic pathogens. They are found on all continents except Antarctica [1]. Generally, each hantavirus species is associated with one species of small mammals acting as their natural reservoir [1]. Human pathogenic hantaviruses are transmitted by rodents [2], among which they circulate asymptomatically. Transmission occurs by inhalation of aerosolized urine, droppings, or other organic matter from infected rodents and by rodent bites [3]. In humans, hantaviruses can cause hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) [2, 4].

Several pathogenic hantaviruses have been found in Russia: Puumala, Hantaan, Seoul, and Dobrava-Belgrade viruses (Kurkino and Sochi variants) [5]. The most important hantavirus is the Puumala virus accounting for almost 98% of the total HFRS cases in Russia [5]. It causes a milder form of HFRS with low mortality rates (less than 1%), which is often referred to as nephropatia epidemica in Europe. The natural reservoir host of the Puumala virus is the bank vole, *Myodes glareolus* [6].

Rodent population dynamics depends on climatic conditions affecting seed production and subnivean reproduction capability; therefore, the HFRS incidence varies from year to year [7]. The highest numbers of bank voles live in broad-leaved and coniferous-broad-leaved forests; therefore, in Russia, the most active epidemic foci of Puumala virus-HFRS are in the Urals and the Middle Volga region [5, 7].

The Puumala virus is an enveloped virus with a genome consisting of three single-stranded negative-sense RNA segments: small (S), medium (M), and large (L) approximately 1828, 3650, and 6550 nucleotides long, respectively [8].

The Puumala virus splits into 8 genetic lineages: Central European (CE), Alpe-Adrian (ALAD), Danish (DAN), South-Scandinavian (S-SCAN), North-Scandinavian (N-SCAN), Finnish (FIN), Russian (RUS), and Latvian (LAT) [9, 10]. The RUS, FIN, and LAT lineages have a common ancestor and originate from the same refugium [10]. The Puumala virus belonging to RUS and FIN lineages was found in Russia [11, 12]; the virus belonging to the clade known as W-RUS was detected in the Kursk Region [13].

HFRS cases are reported in 52 regions in European Russia [5]. The Volga Federal District (VFD) is the most endemic region for HFRS [14–18]. The Central Federal District (CFD) accounts for nearly 13% of the total HFRS cases in Russia. The number of HFRS cases in CFD, most of which were caused by the Puumala virus, increased approximately by 1/3 compared to the previous decade [19]. Despite the relatively high incidence rates, there are very scarce data on nucleotide sequences of hantaviruses circulating in the above region. In the GenBank (NCBI) database, except for the data from our previous study, we were not able to find complete coding sequences for the S segment, which were isolated in CFD [13]. In this study, we examined viruses, which were isolated from bank voles caught in the Volokolamsk District of the Moscow Region.

Materials and methods

Biological sample collection

Rodents were captured in the Volokolamsk District of the Moscow Region in 2019–2020 using Schipanov traps [20]. The trapping was conducted in three stages: 42 voles were captured from June 17 through June 18, 2019 (Novopavlovskoe village, 55.935984°N, 36.169937°E, 200 trap-days), 26 voles from September 18 through September 19, 2019 (Novopavlovskoe village, 90 trap-days), and 41 voles from July 28 through August 4, 2020 (Suvorovo village, 56.127875°N, 35.871444°E, and Alferyevo village, 450 trap-days). Species of small mammals were identified morphologically. Then, liver, spleen, kidney, and lung tissue samples were collected aseptically from the euthanized animals. The study was performed in compliance with the standard practices for safe handling and sample collection from small mammals that were potentially infected with pathogens [21].

Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с Consensus author guidelines for animal use (IAVES 23 July 2010). Протокол исследования одобрен Локальным этическим комитетом (Протокол № 92 от 20 мая 2019 г.).

RNA extraction

For RNA extraction, we used 10% lung tissue suspension in PBS buffer and the RIBO-prep reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Moscow) according to the manufacturer's instruction.

Amplification and sequencing

For cDNA synthesis, we used the Reverta L kit from the Central Research Institute of Epidemiology of Rospotrebnadzor according to the manufacturer's instruction. All samples were further tested by the nested polymerase chain reaction (PCR) method using genus-specific primers amplifying the L-segment region [22]. The resulting amplicons were sequenced using Sanger sequencing with internal nested PCR primers.

To obtain long sequences of three segments using the previously published primers [13], we generated amplicons approximately 1200 nucleotides long with overlapping regions of about 500 nucleotides.

For each sample, the amplicons were pooled in equimolar ratios. To prepare indexed libraries, we used the Nextera XT Library Prep Kit (Illumina) according to the manufacturer's instruction. DNA fragments ranging from 200 bp to 400 bp were removed using Agencourt AMPure XP magnetic beads (Beckman Coulter, United States). The length range of fragments was measured with the Agilent High Sensitivity DNA Kit (Agilent Technologies, Germany) using the Agilent 2100 bioanalyzer. Concentrations of the libraries were measured by quantitative PCR (NEBNext Library Quant Kit for Illumina, New England Biolabs, United Kingdom). The normalized libraries were sequenced using a MiSeq instrument and MiSeq Reagent Kit v3.

Data preprocessing

The fastq files were preprocessed using Trimmomatic v0.39 [23] and cutadapt v3.4 software [24]. The reads were aligned with references using the bowtie2 v2.4.4 tool [25]. Puumala virus NC_005224.1, NC_005223.1, NC_005225.1 sequences were used as reference sequences for S, M, and L segments, respectively. Consensus sequences were generated consecutively 2 times – first, using Lofreq v2.1.5 [26] and bcftools consensus v1.13 [27]; then, the generated consensus sequences were used as reference sequences in bowtie2. The second stage of consensus assembly was performed using GATK HaplotypeCaller v4.2.0.0 [28] and bcftools consensus.

Phylogenetic analysis

The MEGA X software was used for building phylogenetic trees and calculating genetic distances [29]. Phylogenetic trees were constructed using the maximum likelihood method with the general time-reversible (G+I) model. Other Puumala virus sequences from the GenBank database were added to the new sequences.

Results

A total of 109 rodents were captured at trapping locations (**Table 1**). Among the small mammals captured in mixed woodlands, bank voles *M. glareolus* prevailed, while rodents trapped in meadows and brushwood were mostly represented by pygmy wood mice (*A. uralensis*) and yellow-necked mice (*A. flavicollis*).

Among samples from the captured animals, the PCR test detected 6 samples from *M. glareolus* containing hantavirus RNA. Three positive voles were captured near Novopavlovskoye village, two voles were captured in the surroundings of Suvorovo village, and one vole was captured in Alferevo village. The infected bank voles accounted for 7.3%.

The positive samples were sequenced with primers flanking a 347 bp fragment of the L gene. **Table 2** shows identifiers of the generated sequences in the GenBank database. All sequences belonged to Puumala virus.

Long sequences of all segments were generated for samples No. 57 and 79 from the Novopavlovskoye village and Suvorovo village area (Table 2). For further construction of dendrograms, representatives of the Puumala virus, which belonged to the RUS genetic lineage, were added to the generated sequences. Representatives of other lineages comprised an outgroup. The phylogenetic trees were based on alignments of S segment (**Figure** *a*), M segment (Figure *b*), and L segment (Figure *c*). Each sequence was labeled with the name of the isolation region and the genetic lineage.

The phylogenetic trees demonstrate that for all three segments, the genetic variants from the Volokolamsk district group with the sequences from the Kursk Region, which were earlier presented as the W-RUS sublineage [13].

Differences in nucleotides among the sequences from the Volokolamsk district did not exceed 4% for each segment, and they were less than 10% different from the genetic variants from the Kursk Region. **Table 3** shows genetic distances between the generated sequences and genetic variants of the Puumala virus from other locations. The AF284343 isolate was excluded from the analysis due to its insufficient length.

Discussion

Based on the statistics from Rospotrebnadzor, HFRS cases are reported in CFD every year. The largest number of cases are reported in Yaroslavl, Ryazan, Kostroma, and Tula regions. The Puumala virus is responsible for most of the cases, while the Dobrava-Belgrade virus accounts for 3-5% of the total cases [19]. In the meantime, the genetic variants circulating within CFD are poorly studied: In addition to the sequences from the Kursk Region, which had been generated in our previous study [13], the GenBank database had only two sequences: AF284343 [30] and EU652421 [31] at the time we wrote this article [31].

The AF284343 sequence was obtained from the bank voles captured in the Yegorievsky district of the Moscow Region during the Puumala-HFRS outbreak in 1995. The isolate was identified as a new genotype of the Puumala virus [30]. It was a fragment of the S segment, about 700 nucleotides long; on the dendrogram (Fig. a), it clusters with sequences from the Kursk Region and Volokolamsk district. Therefore, apparently, this isolate can be assigned to the W-RUS sublineage.

EU652421 is a short (fewer than 300 nucleotides) fragment of the M segment of the viral RNA, which was isolated from bank voles in the Lipetsk Region. It does not have the length sufficient to construct a well-grounded dendrogram; therefore, it is impossible to identify whether it belongs to the W-RUS sublineage.

Our study has confirmed that the Puumala circulates in the Moscow Region [30]. New sequences from Volokolamsk cluster with the genetic variants from the Kursk Region, which were previously designated as the W-RUS clade [30], for each of the three genomic segments (Fig. 1), thus showing that they belong to the same clade. This statement is supported by the fact that there are fewer genetic differences between the sequences from the Volokolamsk district and the sequences from the Kursk Region than differences between the sequences from the Volokolamsk district and those of other representatives of the RUS lineage (Table 3).

Apparently, studies on systematization of the Puumala

Table 1. Results of trapping of rodents and researching samples for the presence of hantaviral RNA
Таблица 1. Результаты отлова грызунов и исследования образцов на наличие РНК хантавирусов

Location Локация	Biotope Биотоп	Trap-days Ловушко-суток	Количество ПЦР-положительных особей/пойманных особей PCR-positive specimens/Trapped specimens						
			all всего	*Myodes glareolus*	*Microtus oeconomus*	*Microtus* sp.	*Apodemus flavicollis*	*Apodemus uralensis*	*Apodemus agrarius*
Novopavlovskoe Новопавловское	Mixed forest Смешанный лес	290	3/68	3/59	0/1	–	0/6	0/2	–
Suvorovo Суворово	Mixed forest Смешанный лес	200	2/21	2/16	–	–	0/2	0/2	0/1
Alferevo Алферьево	Meadow, shrubs Луг, кустарники	250	1/20	1/7	–	0/2	0/1	0/6	0/4
All Всего		740	6/109	6/82	0/1	0/2	0/9	0/10	0/5

Note. «–» – were absent in the trapping.

Примечание. «–» – отсутствовали в отловах.

Table 2. Data on the Puumala virus sequencing results

Таблица 2. Данные о результатах секвенирования вируса Пуумала

Зоологический номер ID	Isolation point Место изоляции	Isolate name Название изолята	Segment Сегмент	Length, bp Длина, п.н.	ID GenBank
45	Novopavlovskoye Новопавловское	Volokolamsk/Mg45	L	347	OQ503846
57	Novopavlovskoye Новопавловское	Volokolamsk/Mg57	L M S	5972 3058 1779	OQ606916 OQ606917 OQ606918
59	Novopavlovskoye Новопавловское	Volokolamsk/Mg59	L	347	OQ503847
79	Suvorovo Суворово	Volokolamsk/Mg79	L M S	6052 3543 1763	OQ606919 OQ606920 OQ606921
93	Suvorovo Суворово	Volokolamsk/Mg93	L	347	OQ503849
123	Alferevo Алферьево	Volokolamsk/Mg123	L	347	OQ503850

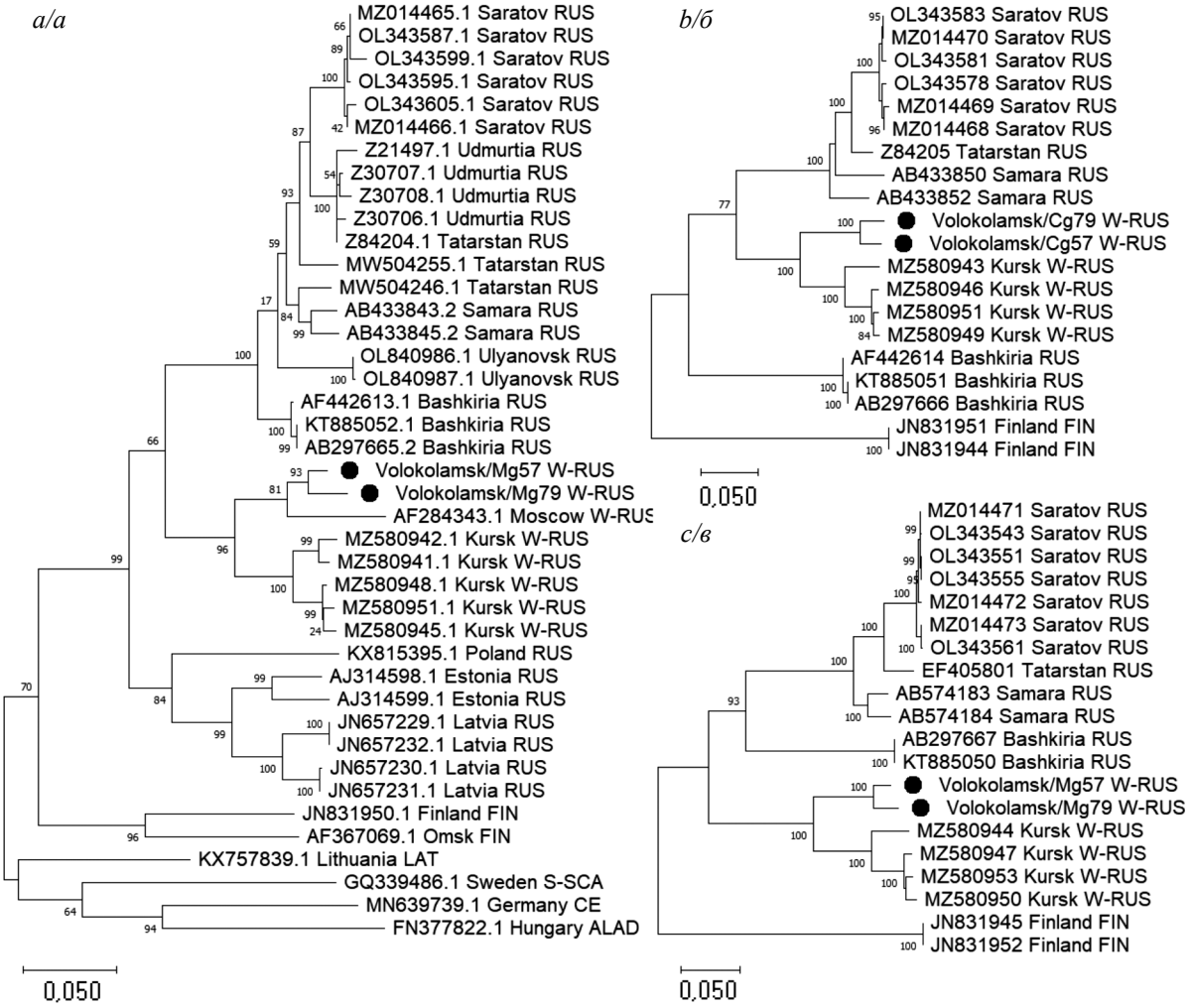

Figure. Phylogenetic trees constructed on the basis of alignments: *a* – 1302 nucleotides of the S segment corresponding to the complete reading frame of the nucleocapsid protein; *b* – 3050 nucleotides of the M segment (partial open reading frame, ORF); *c* – 5960 nucleotides of the L segment (partial ORF). The sequences obtained in this study are marked. Phylogenetic trees were constructed using the Maximum Likelihood method using the General Time Reversible (G+I) model using the MEGA X program [29].

Рисунок. Филогенетические деревья, построенные на основании выравниваний: *а* – 1302 нуклеотидов S-сегмента, соответствующие полной рамке считывания белка нуклеокапсида; *б* – 3050 нуклеотидов M-сегмента (частичная открытая рамка считывания, ОРС); *в* – 5960 нуклеотидов L-сегмента (частичная ОРС). Последовательности, полученные в этом исследовании, выделены маркировкой. Конструирования филогенетических деревьев проводили методом Maximum Likelihood с применением модели General Time Reversible (G+I) с помощью программы MEGA X [29].

Table 3. P-distance between nucleotide/amino acid sequences from Volokolamsk region and sequences from other locations (%)

Таблица 3. Генетическая дистанция между нуклеотидными/аминокислотными последовательностями из Волоколамского района и последовательностями из других локаций (%)

Parameter Параметр	Volokolamsk region Волоколамский район		
	S segment, 1302 bp S-сегмент, 1302 п.н.	M segment, 3050 bp M-сегмент, 3050 п.н.	L segment, 5960 bp L-сегмент, 5960 п.н.
Volokolamsk region Волоколамский район	3,31 0,69	3,49 1,19	3,26 0,45
Kursk region Курская область	8,3–9,38 1,15–1,62	9,04–9,54 1,28–1,58	9,47–9,66 1,41–1,61
Other RUS lineage representatives Другие представители генетической линии RUS	10,91–13,91 1,62–3,93	12,69–13,94 1,87–3,46	13,01–14,14 1,06–1,56

Note. The calculation was performed according to the complete ORF for the S segment, and partial ORF for the M and L segments.

Примечание. Расчет осуществляли по полной ОРС для S-сегмента и по частичным ОРС для M- и L-сегментов.

virus diversity focused on sequences of the S segment [11] due to its better representation in GenBank and its higher conservation.

Sironen et al. (2001) [12] mention that the RUS genetic lineage comprises two sublineages formed by strains from Baltic countries and European Russia. Our study shows that on the phylogenetic tree, the RUS genetic sublineage is divided into three monophyletic groups based on the S segment. The third clade, W-RUS, was described in our previous work and was supplemented with the sequences from the Volokolamsk district in this study.

The variability within the RUS genetic lineage is high compared to other genetic lineages [8]. The recent study addressed systematization and division of the RUS genetic lineage – most represented in GenBank and associated with VFD – into several sublineages [15]. The W-RUS clade is an outgroup in relation to all the described virus clades in VFD, according to the phylogenetic study based on S and L segments (Fig. a, c).

Note that on the dendrogram, the topology of clades for the M segment differs significantly from those for S and L segments (Fig.). This is typical of different types of hantaviruses, as their genomic M segment is characterized by the highest variability and the highest participation in reassortment [8, 9, 32, 33]. The aforesaid supports the previous conclusion [13] that the exchange of M segments had been part of the RUS lineage evolution.

Thus, representatives of the W-RUS clade were found in two regions: Kursk and Moscow Regions. Therefore, it can be assumed that they can also be found in adjacent areas: in Orlov, Bryansk, Kaluga, and Tula Regions. For the complete picture of the genetic diversity of the Puumala virus circulating in European Russia, further studies are required, covering a wider geographic area and using larger sizes of field-collected and clinically tested samples for assessment of the genetic landscape of the Puumala virus found in this area.

Conclusion

Puumala virus RNA of the W-RUS sublineage was detected in rodents in the Volokolamsk district of the Moscow Region.

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