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Study of the circulation and properties of the West Nile virus in Russia in 2022

Andrey V. Toporkov, Elena V. Putintseva, Svetlana K. Udovichenko✉, Natalya V. Boroday, Elena V. Molchanova, Olga S. Bondareva, Aleksander S. Antonov

Volgograd Research Institute for Plague Control, Volgograd, Russian Federation

Abstract

Introduction. The relevance of the study is due to the need to assess the real expansion of West Nile fever (WNF) in Russia, analyze the manifestations of epizootic and epidemic processes and study the population structure of West Nile virus (WNV).

Aim. To obtain objective data on the intensity of WNV circulation in certain territories of Russia and study the properties of the isolated strains of the pathogen.

Materials and methods. 4564 samples of field material from 23 subjects of the Russian Federation and 1547 samples of clinical material from 12 subjects of the Russian Federation were examined. A set of laboratory diagnostic methods was used: ELISA, RT-PCR, virological, sequencing.

Results. In 20 out of 1331 (1.5%) examined febrile patients, IgM antibodies to WNV were detected, including positive cases identified for the first time in the Karachay-Cherkess Republic, Tver and Vladimir regions. The presence of immunity to WNV was found on average in 8.6% of 11 federal subjects' population with high seroprevalence rates in Zaporozhye (24,5%), Tula (15,4%) and Kursk (11,1%) regions. Low-avidity IgG antibodies were detected in 44 (33,1%) samples from a population of 9 federal subjects. Intensive circulation of WNV lineage 2 in the epizootic cycle in the southern part of European Russia was confirmed. 12 WNV isolates were obtained, including those isolated for the first time in the Republic of Crimea, the Karachay-Cherkess Republic and the Stavropol Territory. The heterogeneity of circulating WNF causative agent's strains related to the two clades of the WNV lineage 2 formed in 2021 and 2022 was established.

Conclusions. In the course of comprehensive monitoring studies, a new northern border of the WNF range was established, which as of 2022 passes through the territory of the Tver region. Data from seroepidemiological studies, including the detection of low-avidity IgG antibodies, confirm fairly intense, but undiagnosed contact of the population of the European part of Russia with the WNF pathogen. In the southern region of the European part of Russia, WNV genotype 2 predominantly circulates, belonging to at least two clades formed in 2021 and 2022.

Keywords: *West Nile fever, pathogen monitoring, immunity, the properties of West Nile virus strains*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the Volgograd Research Institute for Plague Control (protocol No. 3, April 25, 2022).

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Оригинальное исследование
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Изучение особенностей циркуляции и свойств вируса Западного Нила в России в 2022 году

Топорков А.В., Путинцева Е.В., Удовиченко С.К.✉, Бородай Н.В., Молчанова Е.В., Бондарева О.С., Антонов А.С.

Волгоградский научно-исследовательский противочумный институт Роспотребнадзора, Волгоград, Россия

Аннотация

Актуальность работы обусловлена необходимостью оценки реального распространения лихорадки Западного Нила (ЛЗН) в России, анализа эпизоотического и эпидемического процессов и изучения популяционной структуры вируса Западного Нила (ВЗН).

Цель — получить объективные данные об интенсивности циркуляции ВЗН на отдельных территориях России и изучить генетическое разнообразие и свойства выделенных штаммов возбудителя.

Материалы и методы. Исследовано 4564 пробы полевого материала из 23 субъектов Российской Федерации и 1547 проб клинического материала из 12 субъектов Российской Федерации. Использован комплекс методов лабораторной диагностики: иммуноферментный анализ, полимеразная цепная реакция с обратной трнскрицией, вирусологический, секвенирование.

Результаты. У 20 (1,5%) из 1331 обследованного лихорадящего пациента выявлены IgM к ВЗН, в том числе впервые в Карачаево-Черкесской Республике, Тверской и Владимирской областях. Наличие иммунной прослойки к ВЗН установлено в среднем у 8,6% населения 11 субъектов с высокими значениями серопревалентности в Запорожской (24,5%), Тульской (15,4%) и Курской (11,1%) областях. Низкоавидные IgG-антитела выявлены в 44 (2,8%) пробах у населения 9 субъектов. Подтверждена интенсивная циркуляция ВЗН 2-го генотипа в эпизоотическом цикле на юге европейской части России. Выделены 12 изолятов ВЗН, в том числе впервые в Республике Крым, Карачаево-Черкесской Республике и Ставропольском крае, изучены патогенные свойства вируса для беспородных белых мышей с определением инкубационного периода заболевания, рассчитана ЛД$_{50}$. Установлена неоднородность циркулирующих штаммов возбудителя ЛЗН, относящихся к двум кладам ВЗН 2-го генотипа, сформированным в 2021 и 2022 гг.

Выводы. В ходе комплексных мониторинговых исследований установлена новая северная граница ареала ЛЗН, которая по состоянию на 2022 г. проходит на территории Тверской области. Данные сероэпидемиологических исследований, в том числе с обнаружением низкоавидных IgG-антител, подтверждают достаточно интенсивный, но не диагностированный контакт населения европейской части России с возбудителем ЛЗН. В южном регионе европейской части России преимущественно циркулирует ВЗН 2 генотипа, относящийся как минимум к 2 кладам, сформированным в 2021 и 2022 гг.

Ключевые слова: *лихорадка Западного Нила, мониторинг за возбудителем, иммунная прослойка, штаммы вируса Западного Нила*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен комиссией по биоэтике ФКУЗ «Волгоградский научно-исследовательский противочумный институт» Роспотребнадзора (протокол № 3 от 25.04.2022).

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

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

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Introduction

The wide territorial distribution of West Nile fever (WNF) in Russia, annual registration of disease cases, including epidemic outbreaks, detection of severe forms and fatal outcomes determine the necessity for constant and systematic epidemiological surveillance of this infection [1, 2]. However, the significant reduction in the volume and quality of monitoring studies of WNF pathogen in Russia observed in recent years makes it difficult to conduct an objective analysis of WNF symptoms and to develop a forecast of the epidemiological situation. Thus, the number of examined patients with WNF-like symptoms in 2020 and 2021 decreased by 5.7 and 4.2 times relative to the figures for 2019, and the volumes of zoological and entomological study material decreased by 2.1 and 1.5 times, respectively. During the same period, WNF pathogen markers were detected by practical institutions of Rospotrebnadzor only in 3 and 7 subjects of Russia [3].

Given the above, one of the key objectives of the Reference Center on WNF pathogen monitoring is to conduct active reconnaissance monitoring of WNF, which allows obtaining reliable information on the intensity of epizootic and epidemic processes. Our studies conducted in 2020–2021 against the background of a low level of officially registered WNF incidence in Russia (10 and 6 times lower than the average annual value, respectively) confirmed the ongoing intensive contact with the pathogen among the population of the Republics of Kalmykia, Crimea, Adygea, Krasnodar Territory, Volgograd and Astrakhan regions [3].

There is a serious problem of establishing the range of WNF in Russia, which is justified by the confirmation of local cases of West Nile Virus (WNV) infection only in 26 federal subjects, while in most of the country (48 federal subjects) only fragmentary data have been obtained, indicating signs of immunity to the WNV among the population and/or detection of markers of the pathogen in zoological and entomological material. As of 2022, the circulation of WNV has not been confirmed in the territory of 11 federal subjects.

The study of the population structure and distribution of various genetic lines of WNV in Russia remains a relevant area of research. It is known that the circulation of WNV genotypes 1, 2 and 4 has been confirmed in Russia [1, 4]. A study of the genome structure of the WNF pathogen circulating in the early 2000s in the south of the European part of the country, the south of Western Siberia, and the Far East demonstrated the predominance of WNV genotype 1a [5–7]. Subsequently, circulation of WNV of two epidemically significant genotypes – 1a and 2 with the predominance of the latter, and genotype 4 in the southern regions was demonstrated in a number of territories of the European part of Russia [4, 7]. Nowadays, due to a decrease in the volume of WNF pathogen monitoring studies, there are only limited data on the circulation of WNV genotype 2. At the same time, the discovery in 2021 of the WNV genotype 2 variant, which has never been found in Russia before [3], determines the necessity to strengthen the WNF pathogen monitoring system to obtain information on the properties of circulating strains, to assess their impact on the clinical picture of the disease and specific features of the epidemic process, and to understand the probable ways of virus entry and spread.

The aim of the study is to obtain objective data on the intensity of WNV circulation in certain territories of Russia and study the genetic diversity and properties of isolated strains of the pathogen.

Materials and methods

Active monitoring of the WNF pathogen in the 2022 season was conducted in 23 federal subjects of Russia by the Volgograd Anti-Plague Scientific Research Institute of Rospotrebnadzor in cooperation with specialists from the Center for Hygiene and Epidemiology, research and anti-plague institutions of Rospotrebnadzor. Collection of blood-sucking arthropods, capture of small mammals and birds, preparation of samples for examination were carried out in accordance with Sanitary rules and regulations 3.3686-21, MG 3.1.3.2600-10 and MR 3.1.0211-20. Arthropods were pooled by species, date and place of collection: mosquitoes — from 2 to 30 specimens, ticks — from 1 to 7 specimens.

Blood samples were collected in 11 federal subjects of the Russian Federation in July–August 2022 from febrile patients undergoing outpatient or inpatient treatment for diseases unrelated to WNF (acute respiratory viral infections, COVID-19, somatic diseases, etc.). In the Zaporozhye region, blood serum samples of donors received from the Melitopol Regional Blood Center in October 2022 were used. The study was conducted with voluntary informed consent of patients. The study protocol was approved by the Bioethics Commission of the Volgograd Anti-Plague Scientific Research Institute (protocol No. 3 of 25.04.2022).

Laboratory tests were conducted at the Volgograd Anti-Plague Scientific Research Institute, except for material from the Zaporozhye region and the Republic of Crimea, which was examined in the laboratory of the mobile complex of the Specialized Anti-Epidemic Brigade of Rospotrebnadzor. A total of 1,547 samples of clinical material and 4,564 samples of field material (3,181 samples of mosquitoes of 28 species, 1 sample of blood-sucking midges of 1 species, 2 samples of blood-sucking flies of 1 species, 1,373 samples of ixodes ticks of 15 species, 10 samples of argas ticks of 1 species, 283 samples of bird brain of 29 species, 325 samples of small mammal organs of 14 species) were examined.

Screening of biological material samples for the presence of WNV RNA was performed using the

AmpliSens WNV-FL reagent kit (Central Research Institute of Epidemiology of Rospotrebnadzor), WNV typing — Ampligen-WNV-genotype-1/2/4 (Volgograd Anti-Plague Scientific Research Institute).

The VectoNil-IgG and VectoNil-IgM (Vector-Best) commercial kits were used for detection of IgM and IgG antibodies to WNV in sera. In samples positive for the presence of IgG antibodies, the avidity index was determined using the VectoNil-IgG-avidity kit (Vector-Best). In cases of detection of IgM and/or IgG antibodies to WNV in serum samples from persons living in areas endemic for viral tick-borne encephalitis, paired quantitative studies of antibodies to WNV and tick-borne encephalitis virus (TBEV) were performed. Determination of antibodies to TBEV was performed using VectoTBEV-IgM and VectoTBEV-IgG reagent kits (Vector-Best). In cases of a significant excess of serum antibodies to WNV compared to TBEV or absence of antibodies to TBEV, a decision was made in favor of the specificity of the detected antibodies to WNV.

The sources of information on the number of registered WNF cases were statistical surveillance forms No. 2 "Information on Infectious and Parasitic Diseases" in the Russian Federation for 2009–2022. Information on the level of immunity to WNV among the population of the surveyed territories was the reported data submitted by the Rospotrebnadzor offices in the subjects of the Russian Federation to the Reference Center, and materials of scientific publications.

Virus isolation was performed according to the classical protocol by infecting a monolayer of Vero cell line with the supernatant of biological material samples in which WNV RNA was detected [8]. To achieve sufficient infectious activity, each isolate was passaged three times on the Vero cell line culture. The study of pathogenic properties was carried out in relation to 22 WNV strains isolated from 2018 to 2022 from samples of field material and stored in the working collection of the Volgograd Research Anti-Plague Institute of Rospotrebnadzor (**Table 1**). In order to study the pathogenic properties of these strains, mongrel white mice were injected intramuscularly with 100 μl of ten times-passaged virus-containing material. The animals were monitored and their deaths were recorded for 21 days after infection. The LD_{50} was calculated using Kerber's formula modified by Ashmarin. Statistical processing of the data was performed using the Mann–Whitney criterion.

Total RNA was isolated from the inactivated filtered supernatant of the Vero cell line using the RNeasy Mini Kit, RNeasy Mini Spin Columns kit (Qiagen). Preparation of libraries for sequencing was performed according to the method proposed by L.A. Moser et al. [9]. Sequencing was performed on the Illumina MiSeq platform (Illumina corp.) using the MiSeq Reagent Kit v3 (600 cycle) (Illumina corp.). Sequences were assembled *de novo* and to the reference genome of WNV (GenBank accs. NC_001563.2) using Cutadapt 2.9, Samtools 1.9, Bcftools 1.9, bwa 0.7.17-r1188 and SPAdes v3.15.1 software products, combined into pipelines using custom scripts implemented in Python 3 language. Nucleotide sequences of the complete genomes of WNV isolates with a potential glycolysis site identified at position N1433 of the NS2B protein were deposited in the international GenBank database (https://www.ncbi.nlm.nih.gov/genbank) under registration numbers OP345101, OP345085, OP345086, OP345087, OP345088, OP345089, OP345090, OP345091, OP345092, OP345093, OP345094, OP345098, OP345099, OP345100, OR159869, OQ214888, OR159871, OR159873, OQ214890, OR159872, OQ214891.

Results

When examining 1331 blood serum samples from febrile patients, IgM antibodies to WNV were detected in 20 (1.5%) cases (**Table 2**). Based on the clinical picture of the disease and the results of laboratory examination, all patients whose sera contained IgM antibodies to WNV, indicating the acute phase of the infectious process, were diagnosed with WNF by medical specialists. WNV RNA was not detected in the tested samples.

In the Karachay-Cherkess Republic, Vladimir and Tver regions, local cases of WNF were registered for the first time, which allows us to speak about the established expansion of the area of this infection.

Studies conducted by the Reference Center specialists confirmed human WNV infection in the Tambov region (Table 2), where the first local case of WNF was detected by specialists of a medical organization in April 2022.

In the rest of the Russian regions, the incidence was officially registered earlier. Manifestations of WNF had an outbreak character only in the Lipetsk Region (2012 — 35 patients) with subsequent registration of sporadic cases in 2013 (4), 2015, 2017, 2021 (1 each), 2016 (3), 2019 (6) [10]. In Ulyanovsk Region until 2022, WNF cases were detected in 2009 (1 patient), 2012 (4); in Belgorod Region — in 2012 (5), 2013 (2), 2014 (1); in Kursk Region — in 2012 (1) and 2019 (4); in Tula Region — in 2021 (1); in Samara — in 2012–2014 (9 each), 2015 (4), 2016–2017 (3 each), 2019 (2); in Zaporozhye — in 2009 (11), 2011 (5), 2012 (10), 2018 (8), 2019 (4); in Stavropol Territory — in 2012 (2), 2018 (2), and 2019 (4) [11].

The presence of immunity to WNV was found in an average of 8.6% of the population living in the territory of 11 subjects (Table 2). The data obtained indicate the continued contact of the population with the WNF pathogen and the necessity to strengthen epidemiologic surveillance activities.

When comparing the results of our studies with the average level of immunity to WNV among the population of the surveyed subjects for 2009–2022, similar

Table 1. West Nile virus strains from the collection of the Volgograd Anti-Plague Scientific Research Institute, used in the study

No.	Strain name		Selection source	Location of vector/host
	collection of the Volgograd Plague Control Research Institute	Department of collection of microorganisms, State Research Center of Virology and Biotechnology VECTOR		
1	Volg601/18	V-959	*Culex modestus*	Volgograd Region
2	Volg696/18	V-960	*Culex pipiens*	Volgograd Region
3	Volg723/18	V-961	*Culex pipiens*	Volgograd Region
4	Volg774/18	–	*Culex spp.*	Volgograd Region
5	Volg829/18	V-962	*Culex pipiens*	Volgograd Region
6	Volg840/21	V-1203	*Culex pipiens*	Volgograd Region
7	Voronezh796/21	–	*Culex pipiens*	Voronezh Region
8	Volg701/21	V-1202	*Culex pipiens*	Volgograd Region
9	Astrahan962/21	–	*Culex modestus*	Astrakhan Region
10	Rostov 362/21	–	*Corvus frugilegus*	Rostov Region
11	Krim221/22	–	*Culex pipiens*	Republic of Crimea
12	Krim233/22	–	*Culex pipiens*	Republic of Crimea
13	Krim245/22	–	*Culex pipiens*	Republic of Crimea
14	Klmk499/22	–	*Culex pipiens*	Republic of Kalmykia
15	Klmk 502/22	–	*Culex pipiens*	Republic of Kalmykia
16	Volg 565/22	–	*Culex pipiens*	Volgograd Region
17	KCHR755/22	–	*Culex modestus*	Karachay-Cherkess Republic
18	Volg 912/22	–	*Culex pipiens*	Volgograd Region
19	Volg 911/22	V-1388	*Culex modestus*	Volgograd Region
20	Stavropol1516/22	–	*Culex pipiens*	Stavropol Territory
21	Stavropol1451/22	V-1389	*Culex pipiens*	Stavropol Territory
22	Astrahan1031 /22	–	*Culex pipiens*	Astrakhan Region

seroprevalence rates were found in Stavropol Territory (5.9%), Belgorod (8.1%), Lipetsk (3%), Ulyanovsk (2.9%), and Samara (3.9%) regions. A noteworthy result is the high percentage of positive findings for IgG antibodies to WNV in Tula (15.4%) and Kursk (11.1%) regions, where only a few WNF patients have been observed. As for the Tver Region, the presence of antibodies to WNV in the local population during routine monitoring was detected once in 2014 (1.3%), in the Tambov Region — in 2012 (2.6%), in the Vladimir Region — in 2016 (3%). There were no official reported data on the immunity to WNV among the population of the Karachay-Cherkess Republic and Zaporozhye Region.

In the samples positive for IgG antibodies, avidity was assessed in order to determine the age of contact with the pathogen. It was found that 44 blood serum samples from the population of 9 subjects contained low-avid IgG antibodies, indicating infection with WNV in the previous 2–3 months.

Circulation of WNV in the epizootic cycle in 2022 in the south of European Russia was confirmed in all the surveyed territories, except for the Republic of Adygea (**Table 3**). A high proportion of positive findings among blood-sucking mosquitoes was found in Stavropol Territory (8.2%), Volgograd Region (5.9%) and Karachay-Cherkess Republic (3.8%), which indicated a significant epidemiologic risk for the population there.

In the oldest WNF focus, Astrakhan Region, while the percentage of PCR-positive samples of bloodsucking mosquitoes was low (0.3%), a high proportion of positive findings (3.2%) was found in samples of ixodid ticks. The detection rate of WNV among ixodid ticks of *H. marginatum* species taken from cattle was 16% (4 out of 25 samples).

Table 2. The total results of serosurvey for the antibodies to WNV in certain territories of European part of Russia

No.	Name of the constituent entity	Number of samples	Detection of antibodies to WNV					
			IgM		IgG		low avidity IgG	
			n	%	*n*	%	*n*	%
1	Karachay-Cherkess Republic	105	1	0,9	6	5,7	0	0
2	Tula Region	104	0	0	16	15,4	4	3,8
3	Ulyanovsk Region	146	2	1,4	5	3,4	2	1,4
4	Tver Region	100	7	7,0	3	3,0	1	1
5	Samara Region	101	0	0	6	6,0	1	1
6	Tambov Region	108	4	3,7	1	0,9	0	0
7	Kursk Region	117	2	1,7	13	11,1	6	5,1
8	Stavropol Territory	239	3	1,3	12	5,0	5	2,1
9	Vladimir Region	54	1	1,8	0	0	0	0
10	Belgorod Region	154	0	0	13	8,4	1	0,6
11	Lipetsk Region	103	0	0	5	4,9	1	1
12	Zaporozhye Region	216	–	–	53	24,5	23	10,6
Total		1547	20	1,5	133	8,6	44	2,8

Note. The dash means no studies have been conducted.

In the Volgograd Region, the detection of WNV markers in mosquitoes in 2022 was comparable to the indicators of previous years. However, for the first time in the long-term period of the Reference Center studies, 78.6% of all positive findings in the Volgograd Region were in *Ae. vexans* and *Ae. caspius* mosquitoes collected in late June, which confirmed the active epizootic process and was considered by us as a harbinger of possible epidemiological ill health.

The detection rate of WNV markers of blood-sucking mosquitoes in 2022 in the Republic of Kalmykia amounted to 2.3%. With signs of epizootic process activity almost annually observed in this territory, the disease incidence has not been officially registered here since 2013. The species composition of mosquitoes as potential vectors of WNV in the Republic of Kalmykia remains poorly studied. In 2022, based on the results of the entomological survey by the Reference Center specialists, the species composition of mosquitoes was supplemented with *Anopheles algeriensis* and *Coq. richardii* species.

In the Republic of Adygea in 2022, the infestation of carriers and vectors with WNV has not been determined. Unlike other subjects of southern Russia, where high numbers of *Cx. pipiens* species mosquitoes are maintained due to the presence of water reserves in artificial tanks on household plots, in Adygeya, due to the high level of moisture, such breeding sites are practically absent. One of the competent vectors of WNV, *Cx. modestus*, was not detected here either. However, in the Republic of Adygea we detected *Ae. koreicus* species mosquitoes (Maykop), the presence of which in this area is not described in available publications.

In the Republic of Crimea, the proportion of mosquito samples positive for WNV RNA was 2%, while in urbanized habitats this indicator was significantly higher, reaching 4.1%. The majority of positive findings were made by *Ae. caspius* species mosquitoes, which was the common eudominant species in urbanized and suburban biotopes. All findings in 2022 were detected in vector samples captured in the north of Crimea (Krasnoperekopsk district), where WNV circulation had not been previously established.

Among the subjects of the center of European Russia and Western Siberia, markers of WNF pathogen in zoological and entomological material were detected only in the territory of the Tula Region (Table 3).

Summarizing the data of field studies using the molecular genetic method, we note that in vector samples, WNV RNA was detected predominantly in low concentrations. In 43 (79.6%) of 54 samples, the threshold cycle (Ct) value exceeded 28 and indicated the prevalence of relatively low viral load among infected mosquitoes and ticks. High concentrations of WNV RNA (Ct values: 8.98; 10.2; 14.55; 15.55; 15.77) were detected only in 5 vector samples (4 samples of

Table 3. The volumes and results of laboratory tests for WNV RNA presence in entomological material

No.	Name of the constituent entity	Mosquitoes			Клещи | Ticks		
		number of pools	number of positive pools, %	structure of positive pools	number of pools	number of positive pools, %	structure of positive pools
1	Volgograd Region	474	28 (5,9)	12 — *Aedes vexans*, 10 — *Ae. caspius*, 1 — *Anopheles hyrcanus*, 2 — *Culex modestus*, 3 — *Cx. pipiens*	372	1 (0,3)	1 — *Hyalomma marginatum*
2	Astrakhan Region	338	1 (0,3)	1 — *Cx. pipiens*	124	4 (3,2)	4 — *H. marginatum*
3	Rostov Region	27	1 (3,7)	1 — *Ae. caspius*	21	1 (4,8)	1 — *Ixodes ricinus*
4	Zaporozhye Region	203	6 (3)	6 — *Cx. pipiens*	192	0	
5	Republic of Crimea	693	14 (2)	9 — *Ae. caspius*, 4 — *Cx. modestus*, 1 — *Cx. pipiens*	–	–	–
6	Republic of Kalmykia	174	4 (2,3)	4 — *Cx. pipiens*	95	0	–
7	Republic of Adygea	91	0		57	0	–
8	Karachay-Cherkess Republic	78	3 (3,8)	2 — *Cx. modestus*, 1 — *Cx. pipiens*	7	0	–
9	Stavropol Territory	110	9 (8,2)	9 — *Cx. pipiens*	99	0	–
10	Belgorod Region	18	0	–	0	0	–
11	Kursk Region	166	0	–	59	0	–
12	Lipetsk Region	315	0	–	61		–
13	Tambov Region	20	0	–	11	0	–
14	Tver Region	11	0	–	23	0	–
15	Tula Region	142	1 (0,7)	1 — *Coquillettidia richiardii*	5	0	–
16	Vladimir Region	40	0	–	57	0	–
17	Samara Region	171	0	–	36	0	–
18	Ulyanovsk Region	110	0	–	0	0	–
19	Kemerovo Region	0	0	–	16	0	–
20	Novosibirsk Region	0	0	–	36	0	–
21	Omsk region	0	0	–	12	0	–
22	Khanty-Mansi Autonomous District	0	0	–	28	0	–
23	Tyumen region	0	0	–	72	0	–
Total		3181	67	–	1383	6	–

Cx. pipiens and 1 sample of *H. marginatum*).

According to the results of typing of positive samples, the isolated RNA fragments of WNV were found to belong to genotype 2 in 27 (50%) out of 54 samples: in samples of *Cx. pipiens* mosquitoes caught in the Republic of Kalmykia, Astrakhan Region and Stavropol Territory; *Cx. pipiens*, *An. hyrcanus*, *Cx. modestus*, *Ae. vexans*, *Ae. caspius* mosquitoes in the Volgograd Region; *Ae. caspius* mosquitoes and *I. ricinus* ticks in the Rostov Region. Genotypes 1 and 4 of WNV were not identified in the samples

Genotyping of samples in which the concentration of WNV RNA was below the limit of detection of the Ampligen-WNV-genotype-1/2/4 reagent kit (1×10^4 copies/mL) was difficult. In samples with a Ct value ≥ 30, genotyping was possible in 6 (21.4%) of 28 samples, Ct = 28–30 — 66.7%, while in samples with Ct values < 28 — in 100% of the samples tested.

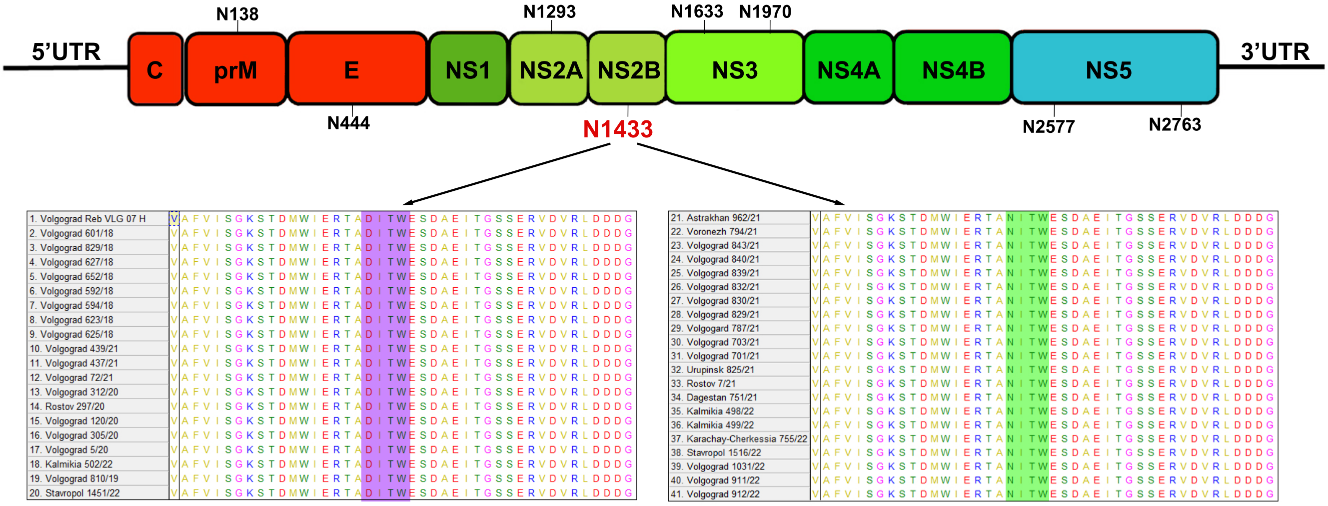
Given the low concentration of the pathogen in the native material, in order to obtain isolates of WNV and study their biological and molecular genetic properties, the virus was accumulated in cell culture. In 2022, 12 WNV isolates were obtained: 3 from the Republic of Crimea, 2 from the Republic of Kalmykia, 1 from the Karachay-Cherkess Republic, 3 from the Volgograd Region, 1 from the Astrakhan Region, and 2 from the Stavropol Territory.

For the strains of WNV isolated in 2022, we have established the features of the course of infection in mongrel white mice with the determination of the incubation period of the disease and calculated the LD_{50}. As a result of infection of laboratory mice, regardless of the used strain of WPN isolated in 2022, the animals developed only influenza-like form of the disease and showed the following symptoms: wet fur, adynamia, chills, drowsiness. In contrast, 20% of mice infected with the strains isolated in 2018 and 2021 developed the disease with signs of nervous system damage in the form of monoplegia and serous meningitis [12].

Animal mortality was observed on average from day 10 to day 14, which, when compared with the data obtained for the strains isolated in 2018 and 2021 (day 3–14 and day 6–14, respectively), suggests a trend towards longer incubation periods. The mean LD_{50} of the strains isolated in 2022 was 10^4–10^5 BOU, while the LD_{50} of the strains isolated in 2018 and 2021 was 10^3–10^4 BOU. Lethality for the strains isolated in 2022 averaged 22% and was statistically significantly different from that of the strains isolated in 2018 (60%) and 2021 (40%) at a significance level of $p = 0.05$. However, when infected with strains from the Astrakhan Region and Stavropol Territory, lethality amounted to 57% and was comparable to previously isolated strains.

Comparative analysis of the full-genome sequences of 12 isolates of WNV showed that 2 of them, isolated in the territory of the Republic of Kalmykia and Stavropol Territory in 2022, form a new distinct clade. The topology of the phylogenetic tree indicates a possible African origin of WNV. This genetic variant was probably introduced into Russia by migratory birds. The remaining 10 isolates belonged to the virus clade formed in 2021 by isolates isolated in Volgograd, Astrakhan, Rostov, Voronezh regions and the Republic of Dagestan. It is noteworthy that in 2022, simultaneous circulation of isolates of both the clade of WNV genotype 2, formed in 2021, and isolates of a new genetic variant was detected in the territory of the Republic of Kalmykia and Stavropol Territory.

We analyzed 44 amino acid complete coding sequences of WNV isolates isolated in 2018–2022 in Russia and performed a comparative analysis with the genotype 2 reference NC_001563, as well as with isolates circulating in Europe. We have identified a number of glycosylation sites that are common to WNV isolates circulating in Russia and European countries. It is known that glycosylation sites play a key role in the interaction of the virus with host cells and they are the so-called hot spots in the adaptive variability of WNV. Also, a potential glycosylation site at position N1433 of the NS2B protein was identified for the first time in a number of isolates isolated in 2021 and 2022 in Russia (**Figure**). Interestingly, this mutation is absent both in the genotype 2 reference and in isolates from European countries. The study did not show that isolates possessing this glycosylation site are confined to specific territories or to currently known genetic variants of genotype 2 of WNV.



1. Volgograd Reb VLG 07 H	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
2. Volgograd 601/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
3. Volgograd 829/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
4. Volgograd 627/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
5. Volgograd 652/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
6. Volgograd 592/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
7. Volgograd 594/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
8. Volgograd 623/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
9. Volgograd 625/18	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
10. Volgograd 439/21	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
11. Volgograd 437/21	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
12. Volgograd 72/21	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
13. Volgograd 312/20	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
14. Rostov 297/20	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
15. Volgograd 120/20	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
16. Volgograd 305/20	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
17. Volgograd 5/20	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
18. Kalmikia 502/22	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
19. Volgograd 810/19	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG
20. Stavropol 1451/22	VAFVISGKSTDMWIERTADITWESDAEITGSSERVDVRLDDDG

21. Astrakhan 962/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
22. Voronezh 794/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
23. Volgograd 843/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
24. Volgograd 840/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
25. Volgograd 839/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
26. Volgograd 832/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
27. Volgograd 830/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
28. Volgograd 829/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
29. Volgogard 787/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
30. Volgograd 703/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
31. Volgograd 701/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
32. Urupinsk 825/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
33. Rostov 7/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
34. Dagestan 751/21	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
35. Kalmikia 498/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
36. Kalmikia 499/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
37. Karachay-Cherkessia 755/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
38. Stavropol 1516/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
39. Volgograd 1031/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
40. Volgograd 911/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG
41. Volgograd 912/22	VAFVISGKSTDMWIERTANITWESDAEITGSSERVDVRLDDDG

Results of the analysis of glycosylation sites of WNV genetic variants circulating in Russia.

Glycosylation sites identified using NetNGlyc-1.0 are indicated by coordinates on the map of the WNV genome. The green color indicates the glycosylation site in a number of isolates identified in the period from 2021 to 2022. The NS2B protein sequence of isolates lacking this glycosylation site is highlighted in purple.

Discussion

Laboratory-confirmed cases of WNF in the Tver and Vladimir regions are the first evidence of local transmission of WNV in these territories. It cannot be excluded that cases of the disease among the population here could have occurred earlier but were not diagnosed by medical specialists, since only a few patients have been tested for WNF markers in these subjects. Our assumption can be supported by the data on immunity to WNF among residents of the Vladimir Region (3% in 2015), as well as the confirmation in 2021 of autochthonous cases in the Moscow Region [13]. In the Tver Region, negative results were obtained according to the data from previous serological and epidemiological studies (2013) [14].

In the territory of the Karachay-Cherkess Republic, WNV circulation has not been established until 2022, which is probably due to the lack of comprehensive monitoring of the WNF pathogen. At the same time, the incidence, including outbreaks, has been confirmed in the neighboring territories — Krasnodar and Stavropol. Summarizing our data on local case detection, vectors infected with WNV and immunity among the population, the territory of the Karachay-Cherkess Republic was proved to be endemic for WNF.

The failure to detect WNV RNA in clinical samples is likely to be due to the fact that the material was collected for testing late in the course of the disease, when the concentration of the virus in the blood decreases sharply. According to the data submitted to the Reference Center by the Rospotrebnadzor offices in the constituent entities of the Russian Federation, the average period of sampling from the onset of clinical symptoms was 6 days, and the maximum was 14 days. The short duration of viremia can be inferred from the course of the disease, which in 85% of patients was mild and characterized by short-term fever.

The values of seroprevalence to WNV obtained by the Reference Center in the Ulyanovsk, Lipetsk, Belgorod, Samara, and Stavropol regions are generally comparable to the values detected during routine serological monitoring. In Kursk Region, the level of immunity to WNV was significantly higher than the indicators established in 2017–2021 during serologic monitoring by Rospotrebnadzor institutions (0.5–1.3%). A similar pattern was noted for the Tula Region, where negative results were obtained in serosurveys performed among healthy population in certain years. However, according to scientific publications, the etiologic role of WNV was confirmed here in 4 febrile patients hospitalized in medical organizations of Tula back in the summer of 2012, and IgG antibodies were detected in 1.5% of those examined [15]. Thus, the seroprevalence of the population of these territories established by us can characterize the high degree and frequency of their contact with the WNF pathogen in the season of 2022.

It is interesting to note that the level of population immunity to WNV in the Zaporozhye Region was comparable to the indicators obtained in the territories of Russia with high epidemic danger, such as Volgograd, Rostov, Astrakhan regions. So far, data on the spread of WNF in Zaporozhye Region have been limited. In available sources there are reports on sporadic cases disease, and there is no information about the immunity of the population to the WNF pathogen in the modern period.

The intensity of the epizootic process in almost all the surveyed territories in the south of European Russia did not correspond to the number of officially registered cases of the disease, which did not exceed 1–3 WNF patients per subject. Apparently, this observation may be associated with a decrease in the efficiency of detection of WNF patients by specialists of medical organizations. The total number of patients with symptoms similar to WNF in 2022 in the territories of the south of European Russia amounted to 542 people, which is 2.8 times lower than the annual average (1,516 people). At the same time, in certain federal subjects of southern Russia (Stavropol Territory, Karachay-Cherkess Republic) in 2022, medical specialists did not actively identify WNF patients.

The high proportion of PCR-positive samples among *H. marginatum* ixodid ticks in the territory of the Astrakhan Region in our study and the data of scientific publications on WNV infestation of 2.6% of *H. marginatum* collected from cattle in the middle delta of the Volga River, as well as detection of WNV RNA in hungry imago collected in early spring, allow us to conclude about their significant participation in the epizootic process and preservation of the pathogen during the inter-epizootic period [16]. The role of this tick species in the epidemic process has not been studied, and this seems to be an actual direction of scientific research.

The high proportion of positive findings among mosquitoes of the genus *Aedes* indicates their important role not only in the course of the epizootic process of WNF, but also, possibly, in the infection of human WNV. However, to understand the epidemiological significance of mosquitoes of the *Aedes* genus or other genera, comprehensive studies are needed to investigate their vector competence, as well as the relationship between the dynamics of vector abundance and the morbidity of the population during the transmission season.

The detection of the Asian *Ae. koreicus* species in Adygea fits into the general trend of expansion of the range of this vector species observed in recent years in southern Russia. The presence of *Ae. koreicus* was established in 2013 and 2018 in Krasnodar Territory, in 2018 — in the Republic of Crimea [17, 18]. The vector competence of this species with respect to WNV in Russia has not been investigated.

Low detectability of WNV markers in the study of zoological and entomological material collected in central Russia could probably be associated with a complex of factors affecting its volume and quality (unfavorable climatic conditions at the time of material collection, choice of biotopes, conditions of collection, storage and transportation of material, etc.). At the same time, the results of the examination of feverish patients presented above testify to the intensive circulation of WNV in the central part of Russia and contact of the population with it.

The lack of positive findings in field samples from Western Siberia is also due to the insufficient volume for the detection of WNV markers, which averaged 33 samples from one subject. In 2021, with a significantly larger volume of material examined (135 samples from the Omsk Region), WNV RNA was detected in 1 sample of *Coq. richiardii* mosquitoes and 2 samples of *I. persulcatus* ticks. Data on the circulation of WNV in Western Siberia are limited to the Omsk, Novosibirsk, Tomsk regions, Krasnoyarsk and Altai Territories [19, 20]. For an objective assessment of the WNF situation in Western Siberia, it is necessary to conduct targeted studies that will provide up-to-date information on the fauna of potential participants in the enzootic cycle of WNV and the activity of WNF foci in these territories.

As a result of the study of pathogenicity of WNV strains isolated in 2022, when infecting white mice with them, the majority of mice showed an increase in the incubation period of the disease and a decrease in lethality compared to the data obtained for strains isolated in other years. Strains from the Astrakhan Region and Stavropol Territory were an exception, being highly virulent for laboratory animals and characterized by a lethality rate of 57%. Similar studies conducted with strains isolated in Australia, the USA, Canada, and Europe also noted the fact of circulation of WNV strains that differed significantly in virulence and neuroinvasive properties [21, 22].

The heterogeneity of WNV strains in terms of pathogenicity is due to their genetic diversity and is a consequence of adaptation to the change of hosts and vectors in the enzootic cycle, in which the virus replicates in cells of evolutionarily distant species.

Conclusions

1. In the course of comprehensive monitoring studies, a new northern border of the WNF range was established, which as of 2022 passes through the territory of the Tver region.
2. Data from seroepidemiological studies, including the detection of low-avidity IgG antibodies, confirm fairly intense, but undiagnosed contact of the population of the European part of Russia with the WNF pathogen.
3. In the southern region of the European part of Russia, WNV genotype 2 predominantly circulates, belonging to at least two clades formed in 2021 and 2022. respectively.

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Information about the authors

Andrey V. Toporkov — D. Sci. (Med.), Associate Professor, Director, Volgograd Research Institute for Plague Control, Volgograd, Russia, https://orcid.org/0000-0002-3449-4657

Elena V. Putintseva — Cand. Sci. (Med.), leading researcher, Laboratory of epidemiological analysis and epizootological monitoring, Volgograd Research Institute for Plague Control, Volgograd, Russia, https://orcid.org/0000-0002-9368-6165

Svetlana K. Udovichenko✉ — Cand. Sci. (Med.), leading researcher, Laboratory of epidemiological analysis and epizootological monitoring, Volgograd Research Institute for Plague Control, vari2@sprint-v.com.ru, Volgograd, Russia, https://orcid.org/0000-0001-8682-1536

Natalya V. Boroday — senior researcher, Laboratory of epidemiological analysis and epizootological monitoring, Volgograd Research Institute for Plague Control, Volgograd, Russia, https://orcid.org/0000-0002-2076-5276

Elena V. Molchanova — Cand. Sci. (Biol.), senior researcher, Laboratory of arbovirus infections, Volgograd Research Institute for Plague Control, Volgograd, Russia, https://orcid.org/0000-0003-3722-8159

Olga S. Bondareva — Cand. Sci. (Med.), senior researcher, Laboratory of genodiagnostics particularly dangerous infections, Volgograd Research Institute for Plague Control, Volgograd, Russia, https://orcid.org/0000-0001-5690-6686

Aleksander S. Antonov — researcher, Laboratory of bioinformation analysis, Volgograd Research Institute for Plague Control, Volgograd, Russia, https://orcid.org/0000-0001-9342-7211

Author contribution: *Toporkov A.V.* — the concept and design of the study; *Putintseva E.V., Udovichenko S.K.* — concept and design of the study, data collection and processing, organizing the collection of clinical material, writing the text of the manuscript, interaction with the institutions of Rospotrebnadzor; *Boroday N.V.* — collection of field material, data processing; *Molchanova E.V.* — conducting virological research, writing the text of the manuscript; *Bondareva O.S.* — conducting molecular genetic research, writing the text of the manuscript; *Antonov A.S.* — carrying out full genome sequencing, writing the text of the manuscript. All authors made a final approval of the version to be published.

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Информация об авторах

Топорков Андрей Владимирович — д.м.н., доцент, директор Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, https://orcid.org/0000-0002-3449-4657

Путинцева Елена Викторовна — к.м.н., в.н.с. лаб. эпидемиологического анализа и эпизоотологического мониторинга Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, https://orcid.org/0000-0002-9368-6165

Удовиченко Светлана Константиновна✉ — к.м.н., в.н.с. лаб. эпидемиологического анализа и эпизоотологического мониторинга Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, vari2@sprint-v.com.ru, https://orcid.org/0000-0001-8682-1536

Бородай Наталья Владимировна — с.н.с. лаб. эпидемиологического анализа и эпизоотологического мониторинга Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, https://orcid.org/0000-0002-2076-5276

Молчанова Елена Владимировна — к.б.н., с.н.с. лаб. арбовирусных инфекций Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, https://orcid.org/0000-0003-3722-8159

Бондарева Ольга Сергеевна — к.м.н., с.н.с. лаб. генодиагностики особо опасных инфекций Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, https://orcid.org/0000-0001-5690-6686

Антонов Александр Сергеевич — н.с. лаборатории биоинформационного анализа Волгоградского научно-исследовательского противочумного института, Волгоград, Россия, https://orcid.org/0000-0001-9342-7211

Участие авторов: *Топорков А.В.* — концепция и дизайн исследования; *Путинцева Е.В., Удовиченко С.К.* — концепция и дизайн исследования, сбор и обработка данных, организация сбора клинического материала, взаимодействие с учреждениями Роспотребнадзора, написание текста рукописи; *Бородай Н.В.* — сбор полевого материала, обработка данных; *Молчанова Е.В.* — проведение вирусологических исследований, написание текста рукописи; *Бондарева О.С.* — проведение молекулярно-генетических исследований, написание текста рукописи; *Антонов А.С.* — проведение полногеномного секвенирования, написание текста рукописи. Все авторы прочли и одобрили финальную версию до публикации.

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