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Lethal cases of lyssavirus encephalitis in humans after contact with bats in the Russian Far East in 2019–2021

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Introduction. On the territory of Russia four species of lyssaviruses (genus *Lyssavirus*) were identified, three of them caused human deaths.
The aim of work: to characterize fatal cases in humans after contacts with bats in the Far East in 2018–2021 and to perform typing of isolated pathogens.
Materials and methods. Lyssavirus infection was confirmed in samples of sectional material from people who died in the Amur Region in 2019, in the Primorsky Krai in 2019 and 2021. Diagnostics was performed by fluorescent antibody test (FAT) and RT-PCR using diagnostic kits of domestic production. Viruses were isolated in a bioassay. The nucleoprotein sequences were analyzed after 1st passage. The analysis of phylogenetic relationships and the construction of a dendrogram were performed using the MEGA7 software.
Results. The viruses that caused the fatal cases in humans in the Amur Region and Primorsky Krai share more than 90% identity to *Lyssavirus irkut* detected in Russia and China. Together they form a separate monophyletic cluster with 100% bootstrap support.
Conclusion. On the territory of Russia, monitoring of bat populations for infection with lyssaviruses is relevant. The material of people who died from encephalomyelitis of unknown etiology within 10–15 days from the onset of the disease must be examined for lyssavirus infection. It is necessary to develop PCR assays that employ genus-specific primers. The use of molecular biological methods is promising for improving the diagnosis of rabies and epidemiological surveillance, as well as increasing the efficiency of the system of biological safety of the population of the Russian Federation.

Keywords: *Russia; rabies lyssavirus; Lysavirus irkut; lyssavirus encephalitis; cheiroptera*

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Случаи летальной лиссавирусной инфекции у людей после контактов с рукокрылыми на Дальнем Востоке России в 2019–2021 гг.

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Введение. На территории России были выявлены четыре вида лиссавирусов (род *Lyssavirus*), представители трёх из них являлись причиной гибели людей.
Цель – охарактеризовать случаи гибели людей после контактов с рукокрылыми на территории Дальнего Востока в 2018–2021 гг. и типировать выделенные патогены.
Материалы и методы. Лиссавирусную инфекцию подтверждали в образцах секционного материала от людей, погибших в Амурской области в 2019 г. и в Приморском крае в 2019 и 2021 гг. Диагностику поводили методом флуоресцирующих антител, Real-time ПЦР, используя диагностикумы отечественного производства. Вирусы выделены в биопробе. Последовательности нуклеопротеина анализировали на уровне 1-го пассажа. Анализ филогенетических отношений и построение дендрограмм выполняли в программе MEGA7.
Результаты. Было установлено, что вирусы, вызвавшие гибель людей в Амурской области и Приморском крае, более чем на 90% идентичны лиссавирусам Иркут (вид *Lyssavirus irkut*), выявленным на территории России и Китая, и образуют с ними отдельный монофилетический кластер со 100%-й бутстреп-поддержкой.
Заключение. На территории России актуален мониторинг популяций летучих мышей на заражённость лиссавирусами. Секционный материал людей, погибших от энцефаломиелита неустановленной этиологии в пределах 10–15 дней от начала болезни, необходимо исследовать на лиссавирусную инфекцию. Требуется разработка ПЦР-тест-систем, включающих родоспецифичные праймеры. Применение молекулярно-биологических методов является перспективным в плане развития диагностики бешенства для совершенствования эпидемиологического надзора и повышения эффективности системы биологической защиты населения Российской Федерации.

Ключевые слова: *Россия; лиссавирус бешенства; лиссавирус Иркут; лиссавирусный энцефалит; рукокрылые*

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Introduction

Lyssaviruses are widespread neurotropic RNA viruses that infect warm-blooded animals and humans, causing fatal encephalitis or rabies (also known as hydrophobia, lyssavirus encephalitis). In humans, infection causes acute encephalitis, usually resulting in death within 10–15 days after the onset of clinical symptoms of the disease. The worldwide distribution of lyssaviruses that are highly pathogenic for humans and mammals, their almost absolute lethality, and absence of treatment for developed disease place lyssaviruses at the top of the agenda for human and animal health [1, 2].

Lyssaviruses belong to the genus *Lyssavirus*, the family Rhabdoviridae and the order Mononegavirales, which currently includes 17 species recognized by the International Committee on Taxonomy of Viruses [3]:

– *Lyssavirus rabies* (rabies virus, RABV);
– *Lyssavirus lagos* (Lagos bat virus, LBV);
– *Lyssavirus mokola* (Mokola virus, MOKV);
– *Lyssavirus duvenhage* (Duvenhage virus, DUVV);
– *Lyssavirus hamburg* (European bat 1 lyssavirus, EBLV-1);
– *Lyssavirus helsinki* (European bat 2 lyssavirus, EBLV-2);
– *Lyssavirus australis* (Australian bat lyssavirus, ABLV);
– *Lyssavirus irkut* (Irkut virus, IRKV);
– *Lyssavirus caucasicus* (West Caucasian bat virus, WCBV);
– *Lyssavirus khujand* (Khujand virus, KHUV);
– *Lyssavirus aravan* (Aravan virus, ARAV);
– *Lyssavirus shimoni* (Shimoni bat virus, SHIBV);
– *Lyssavirus bokeloh* (Bokeloh bat lyssavirus, BBLV);
– *Lyssavirus ikoma* (Ikoma lyssavirus, IKOV);
– *Lyssavirus lleida* (Lleida bat lyssavirus, LLEBV);
– *Lyssavirus gannoruwa* (Gannoruwa bat lyssavirus, GBLV);
– *Lyssavirus formosa* (Taiwan bat lyssavirus, TBLV).

One more virus, the Kotalahti bat lyssavirus (KBLV), remains a tentative species until its species status is confirmed [3]. By 2023, 15 of 17 confirmed species had been isolated from chiropters. *Lyssavirus mokola* and *Lyssavirus ikoma* are the only two lyssaviruses that have not been found among bat species so far [4–7].

Chiropters are a major reservoir of lyssaviruses. Their diversity and global distribution contribute to the biological diversity of pathogens, being highly favorable to the emergence of new species of viruses. Over the last 20 years, 8 new species have been discovered in Europe, Asia, and Africa. Human deaths were reported from 7 species: RABV – about 60,000 deaths a year, resulting from infection from terrestrial mammals, and 2–3 cases a year, resulting from infection from bats in North America; EBLV-1 – 2 cases; EBLV-2 – 2 cases, ABLV – 3 cases, DUVV – 3 cases, IRKV – 1 case, MOKV – 2 cases [8–13].

The rabies lyssavirus (*Lyssavirus rabies*) is the most widespread species, being well represented both geographically and by the species diversity of infected mammals supporting the virus circulation. It circulates in populations of terrestrial mammals, mainly among carnivorous mammals (the order Carnivora) worldwide and in populations of chiropters (the order Chiroptera) in North and South America. *Lyssavirus rabies* is responsible for most of the deaths of humans and animals. Other species of lyssaviruses occur only outside America; they seem to have a limited geographical distribution and a narrower range of hosts. In Europe, including the European part of Russia, 5 species of lyssaviruses have been identified in addition to the rabies lyssavirus; 6 species in Africa, 1 species in Australia, and 5 species have been identified in Asia, including the territory of Russia [7, 11, 12, 14].

The rabies lyssavirus circulates in natural foci across Russia [15, 16], primarily in populations of wild canines (the family Canidae) – foxes, corsac foxes, racoon dogs, wolves, arctic foxes [17–21].

In Russia, two novel lyssaviruses have been discovered among chiropters. The Irkut lyssavirus (*Lyssavirus irkut*) was isolated from a greater tube-nosed bat (*Murina leucogaster*) in the Irkutsk Region in 2002 and the West Caucasian bat lyssavirus (*Lyssavirus caucasicus*) was isolated from a common bent-wing bat (*Miniopterus schreibersii*) in the Krasnodar Territory [22, 23].

Most of the hydrophobia cases reported in the country from 1534 to 2018 were caused by infection through contacts with wild and domestic carnivores (the dog, cat, wolf, fox, racoon dog, etc.) [24]. The molecular and genetic typing of the viruses isolated from the individuals who died after the contacts with the above animals showed that they belonged to the *Lyssavirus rabies* species [15, 25, 26].

Two documented cases of human death, which were associated with bats, were an exception. The first case was reported in the Belgorod Region in 1985. The European bat lyssavirus type 1, presently known as the Hamburg lyssavirus (*Lyssavirus hamburg*), was isolated from a deceased 11-year-old girl [3]. The rabies-related Yuli virus was isolated by professor M.A. Selimov [27] and studied under the supervision of doctor of medical sciences S.V. Gribencha at the Ivanovsky Institute of Virology. The second case was reported in 2007; the Ozernoe lyssavirus, an analog of the Irkut virus, was isolated from a deceased 20-year-old girl in the Primorsky Territory [28].

Another two human deaths caused by bites of chiropters have been reported in Russia. In both cases, the species of lyssaviruses were not identified; the death from lyssavirus infection was diagnosed using clinical and epidemiological data. The first and the earliest reported case of human death occurred in Voroshilovgrad (presently Lugansk, the Lugansk People's Republic of the Russian Federation) in 1977, when a bat bit a 15-year-old girl [29]. The second case was reported in Molodogvardeisk (the Lugansk People's Republic of the Russian Federation) in 2002: A bat bit a 34-year-old man [30].

Thus, out of 4 species of lyssaviruses detected in Russia – *Lyssavirus rabies*, *Lyssavirus hamburg*, *Lyssavirus irkut*, *Lyssavirus caucasicus* – representatives of the first three species caused human deaths. The last three species

were isolated from bats: *Lyssavirus hamburg* was found only in Europe, *Lyssavirus irkut* – in Russia and China, *Lyssavirus caucasicus* – in Russia and Africa [12, 31, 32]. During 1977–2007, four human deaths following bites of chiropters were reported.

In 2018–2021, in the Far East Region of Russia, three new cases of human lyssavirus infection after contacts with bats were reported. These cases are discussed in this article.

The **purpose** of the study was to describe the cases associated with human deaths after contacts with chiropters in the Far East in 2018–2021 and to classify isolated pathogens.

Materials and methods

Lyssavirus infection was confirmed using autopsy samples from patients who died in the Amur Region (Blagoveshchensk) in June 2019, in the Primorsky Territory in September 2019 (Fokino) and in August 2021 (Zavetnoe village). Based on the clinical and epidemiological data, the first two cases were diagnosed with encephalitis of unknown origin (meningoencephalitis of unclear etiology, unspecified viral encephalitis); the last case was diagnosed with acute encephalitis of lyssavirus etiology, severe disease.

The specific rabies virus antigen was detected by the fluorescent antibody test [33] using polyclonal anti-rabies immunoglobulin labeled with fluorescein-5-isothiocyanate (FITC-immunoglobulin) manufactured by the Federal Center for Animal Health (FCAH).

Rus(Amur)8947H_2019, Rus(Primorsky)8949H_2019, and Rus(Primorsky)9220H_2021 viruses were isolated by bioassay using white outbred mice weighing 8–12 g, which were inoculated intracerebrally with 0.03 ml of a 10% Hanks solution-based brain suspension from the deceased patients [34]. The specific death of mice from rhabdovirus was confirmed by detection of its antigen by the fluorescent antibody test performed on impressions of animal brain. The infective activity of the viruses in the native material as well as at the 2nd and 4th passages was measured through intracerebral inoculation of 0.03 ml of brain suspension from the deceased patients in 10-fold dilution in Hanks solution from 10^{-1} to 10^{-7}. The virus titer was calculated using the Reed-Muench method [35]. The authors confirm the compliance with the institutional and national standards for using laboratory animals in accordance with the Consensus Author Guidelines on Animal Ethics and Welfare for Editors (IAVE, July 23, 2010). The studies were approved by the Ethics Committee of the Omsk Research Institute of Natural Focal Infections of Rospotrebnadzor (protocol without number dated Jan 27, 2021).

The real-time RT-PCR (reverse transcription polymerase chain reaction) was performed to detect the rabies virus RNA using reagent kits from Syntol (Moscow) and Fractal Bio (St. Petersburg) and the Rotor-Gene 6000 thermocycler.

The viral RNA was extracted from the brain of the mice that died during the 1st passage, using the TRIzol reagent (Invitrogen Life Technologies, United States) in accordance with the manufacturer's instructions. The synthesis of cDNA (complementary) in RT-PCR was performed using random hexanucleotide primers included in the Reverta-L reverse transcription kit (InterLabService, Moscow).

Overlapping fragments were amplified to identify the primary nucleotide sequence of the nucleoprotein gene fragment [36]. To obtain PCR-fragments of isolated Rus(Amur)8947H_2019, Rus(Primorsky)8949H_2019, Rus(Primorsky)_9220H_2021 viruses, we used pairs of primers described earlier by Heaton et al. [37] for the gene encoding nucleoprotein: JW12-JW6 (DPL), JW6 (M), JW6 (E), and then f1 and r1 described by Liu et al. [38]. The amplification was performed using the Axygen MaxyGene II thermal cycler (Axygen Scientific Inc., United States).

The presence of the PCR product of the required length was detected using electrophoresis in 1.5% agarose gel (1x TAE (tris-acetate buffer)) with ethidium bromide. The purification of PCR products and their concentration were performed using Agencourt AMPure XP paramagnetic beads (Beckman Coulter Life Sciences, United States) in accordance with the manufacturer's recommendations.

The purified PCR product was sequenced using the above pairs of primers and the BigDye™ Terminator v1.1. Cycle Sequencing Kit, followed by the analysis of the reaction products using the SeqStudio genetic analyzer (Thermo Fisher Scientific Inc., United States).

The structure of the resulting chromatograms was analyzed with the help of the Chromas 2.6.6.0 software (Technelysium Pty Ltd, Australia). The obtained fragments of the virus genome were aligned in BioEdit 7.0.5.3 (Informer Technologies, Inc., United States). The length of the obtained products of amplification of nucleoprotein lyssavirus gene fragments was 1258 nt (the genome position – 71-1328 in relation to Reference Sequence NC_001542.1).

The search for homologous nucleotide sequences was performed using the BLASTN 2.12.0+ program in the NCBI database[1].

The analysis of phylogenetic relationships of the obtained nucleoprotein sequences and construction of dendrograms were performed by the neighbor-joining method with MEGA7 software [39], using deposited sequences of *N* gene fragments from the GenBank database ($n = 51$).

The statistical analysis of the results was performed by standard methods using Microsoft Office Excel 2016.

Results

Russia is characterized by the existence of natural foci of rabies, in which the virus circulation is supported by wild carnivores represented primarily by the *Canidae* family [18, 21]. However, since 2019 the situation that is not typical of Russia has been observed: over three years,

[1]Standard Nucleotide BLAST. Available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM = blastn&BLAST_SPEC = GeoBlast&PAGE_TYPE = BlastSearch.

three cases of human death caused by bites of chiropters have been reported – all of them occurred in the Far East.

The first victim of chiropters was a 36-year-old man. In May 2019, he was in his country house in Blagoveshchensk in the Amur Region. When he was putting on a work mitten, he was bitten twice in his ring finger by a chiropter hiding in the mitten. The bite caused minor bleeding. The injured man cleaned out the wound by himself and did not seek any medical care. The disease started developing on the 21st day after the bite. A coma developed on the 10th day of the disease. Death occurred on the 15th day of the disease.

The second victim was a retired man of 73 years old. He was attacked by a bat in Fokino in the Primorsky Territory at the end of August in 2019. The man was working in his garage. The chiropter attacked him and bit him in his neck. He did not go to see the doctor. The incubation period was around 15 days. Death occurred on the 2nd day of the disease.

The third victim was a 35-year-old man. Being at the river in Zavetnoe village in the Chuguev District of the Primorsky Territory in the middle of June 2021, he was bitten by a bat in his upper lip. He cleaned out the wound by himself, seeking no medical care. He became ill on the 52nd day after the bite. A coma developed on the 5th day of the disease. On the 8th day, he died.

In all the victims, the disease developed as meningoencephalitis complicated with hyperthermic syndrome (up to 39–40, 39, and 37.2°C, respectively), intoxication, seizure syndrome, speech disorder, impaired consciousness, pronounced cardiovascular insufficiency, respiratory failure, and brain disorder. Cerebral edema was observed in all the cases. In the first case, there were pulmonary edema and bilateral pneumonia; in the third case – pronounced generalized myoclonia, opsoclonus, myoclonic eyelid retraction, bulbar syndrome, tetraparesis, thrombosis of central retinal veins of both eyes, keratoconjunctivitis. In the first case, the patient was checked for surrogate alcohol poisoning and pneumonia; in the third case – for ischemic stroke.

The characteristics of lyssavirus infection in patients bitten by bats in the Far East Region are summarized in **Table 1**. The species of chiropters that caused infection in the above cases were not identified.

The autopsy material from the deceased was delivered to the Reference Center for Rabies Monitoring at the Omsk Research Institute of Natural Focal Infections of Rospotrebnadzor, following Rospotrebnadzor Order No. 1116 of 1/12/2017. It should be noted that thanks to the vigilance of healthcare workers and the operation of the Reference Center, these cases attracted attention of researchers who were able to identify the etiology of infection.

The material was tested using the real-time PCR reaction and the reagent kit from Syntol (Moscow) for detection of rabies virus RNA. The kit was licensed for medical application. The test system is designed for detection of specific RNA of the rabies lyssavirus (the species *Lyssavirus rabies* – classical rabies virus) by two channels – Yellow and Orange. The recorded increase of the signal in one of the channels is indicative of the presence of specific RNA genome fragments of the above pathogen in the tested sample.

The autopsy material from the patients who died after being bitten by chiropters was tested along with positive samples from wild carnivorous animals.

In samples from all the deceased patients, the positive signal indicating the presence of the rabies pathogen in the autopsy material was received by the yellow channel (**Fig. 1** *a*, *b*, *c*). The presence of pathogens in samples from terrestrial wild carnivores were traditionally recorded as positive by the orange channel (**Fig. 1** *d*).

The test kit from Fractal Bio, which is designed for veterinary purposes and adapted for detection of the classical rabies lyssavirus, did not detect the specific lyssavirus RNA in samples from humans, while all the samples from carnivores were tested positive.

Using the available data proving the circulation of the classical rabies lyssavirus in populations of terrestrial

Table 1. Features of cases of lyssavirus infection in victims of bat bites in the Far East region in 2019 and 2021

Таблица 1. Особенности случаев лиссавирусной инфекции у пострадавших после укусов летучими мышами в Дальневосточном регионе в 2019 и 2021 гг.

Date of contact with source of infection Дата контакта с источником инфекции	Sex, age Пол, возраст	Region Регион	Incubation period Инкубационный период	Clinical period Клинический период	Timely appeal for medical help Своевременное обращение за медицинской помощью
Mid May 2019 Середина мая 2019 г.	Male, 36 years Мужчина, 36 лет	Амурская область, Благовещенск Amur region Blagoveshchensk	21 days 21 дней	15 days 15 дней	Did not apply Не обращался
End of August 2019 Конец августа 2019 г.	Male, 73 years Мужчина, 73 года	Primorsky Krai Fokino Приморский край, ГО ЗАТО Фокино	About 15 days Около 15 дней	2 дня 2 days	Did not apply Не обращался
Mid June 2021 Середина июня 2021 г.	Male, 35 years Мужчина, 35 лет	Primorsky Krai, Chuguevsky District, Zavetnoe village Приморский край, Чугуевский район, с. Заветное	52 days 52 дня	8 days 8 дней	Did not apply Не обращался

carnivores, we assumed that the agent of lyssavirus infection, which was detected by the test kit from Syntol, could belong to the species different from the classical lyssavirus and that the pathogens could belong to the same phylogroup I as the classical lyssavirus.

The specific rabies virus antigen was detected in brain impressions prepared with autopsy material from the deceased, using the immunofluorescence test with polyclonal anti-rabies immunoglobulin from FCAH (**Fig. 2**). The specific fluorescence intensity in two samples (**Fig. 2** *a*, *c*) was graded as 4 (clearly visible yellow-green fluorescence) and in one sample (**Fig. 2** *b*) – as 2 points.

The viruses named Rus(Amur)_8947H_2019, Rus(Primorsky)8949H_2019, and Rus(Primorsky)9220H_2021 were isolated during the bioassay with white outbred mice infected intracerebrally. The incubation period for the Rus(Amur)_8947H_2019 isolate was 10–13 days (on average 11.5 ± 0.3), for Rus(Primorsky)_8949H_2019 – 7–10 days (on average 8.6 ± 0.7), for Rus(Primorsky)_9220H_2021 – 6–8 days (on average 7.1 ± 0.1) (**Table 2**).

All the animals infected with primary material became ill, presenting clinical signs, mostly paralysis and paresis. Other signs included lethargy, weakness, convulsive seizures, decreased activity and mobility. The death of animals occurred within a day. The death of mice from lyssavirus infection was confirmed by detection of a specific antigen in the impressions prepared using the brain of the animals that died or were euthanized when the clinical signs reached the highest level.

The isolated viruses were passaged using white outbred mice weighing 8–12 g. At the 3rd passage, the incubation period for Rus(Amur)8947H_2019 and Rus(Primorsky)8949H_2019 pathogens reduced to 5 days, and for Rus(Primorsky) 9220H_2021 – to 4–5 days. The titer of the Rus(Amur)8947H_2019 virus that was 4.4 lg $LD50_{0.03}$ in the primary material reached 4.7 lg $LD50_{0.03}$ at the 4th passage. For the Rus(Primorsky)8949H_2019 virus, the infective activity was 4.6 lg $LD50_{0.03}$ in the primary material and 5.6 lg $LD50_{0.03}$ at the 6th passage. The titer of the Rus(Primorsky) 9220H_2021 virus in the pri-

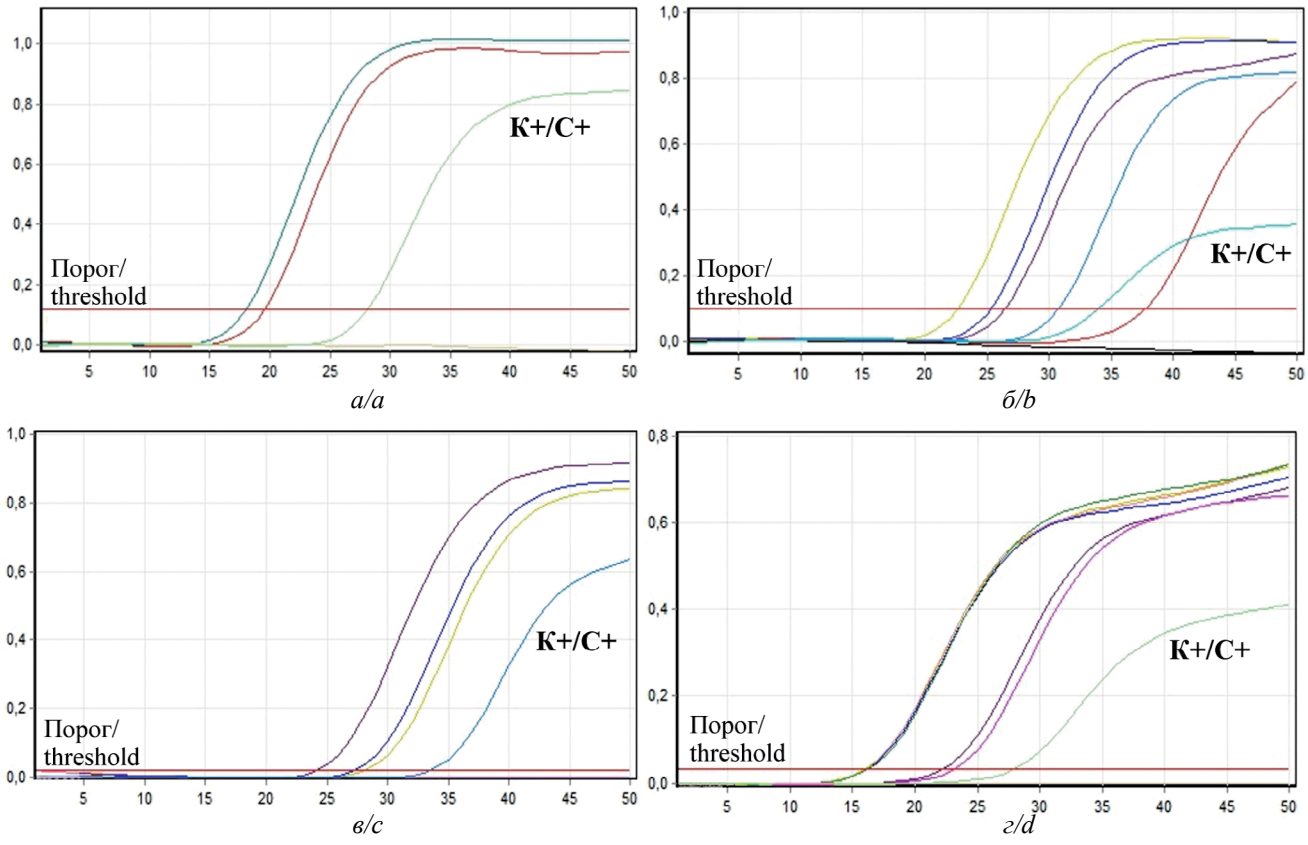

Fig. 1. Results of testing of samples from people who died after being bitten by bats and material of wild carnivores in the Real-time PCR reaction with a set of reagents for the detection of rabies virus RNA from Syntol LLC using specific detection channels: Yellow: *a* – human, Amurskaya region, 2019; *b* – human, Primorsky Krai, 2019; *c* – human, Primorsky Krai, 2021 and Orange: *d* – samples of wild carnivores, Amur Region, 2019. In illustrations *a*, *b*, *c*, *d* curves are to positive reactions for biomaterial samples. C+ – positive reaction control. The X-axis indicates normal fluorescence. The Y-axis indicates the number of cycles.

Рис. 1. Результаты диагностики образов от людей, погибших после укусов рукокрылыми, и материала от диких хищников в ПЦР Real-time с набором реагентов для выявления РНК вируса бешенства от ООО «Синтол» по каналам детекции специфики: Yellow: *а* – человек, Амурская область, 2019 г.; *б* – человек, Приморский край, 2019 г.; *в* – человек, Приморский край, 2021 г. и Orange: *г* – хищные млекопитающие, Амурская область, 2019 г. Кривые на иллюстрациях *а, б, в, г* соответствуют положительным реакциям для образцов биоматериала. К+ – положительный контроль реакции. По оси X указана нормальная флюоресценция. По оси Y указано ко-во циклов.

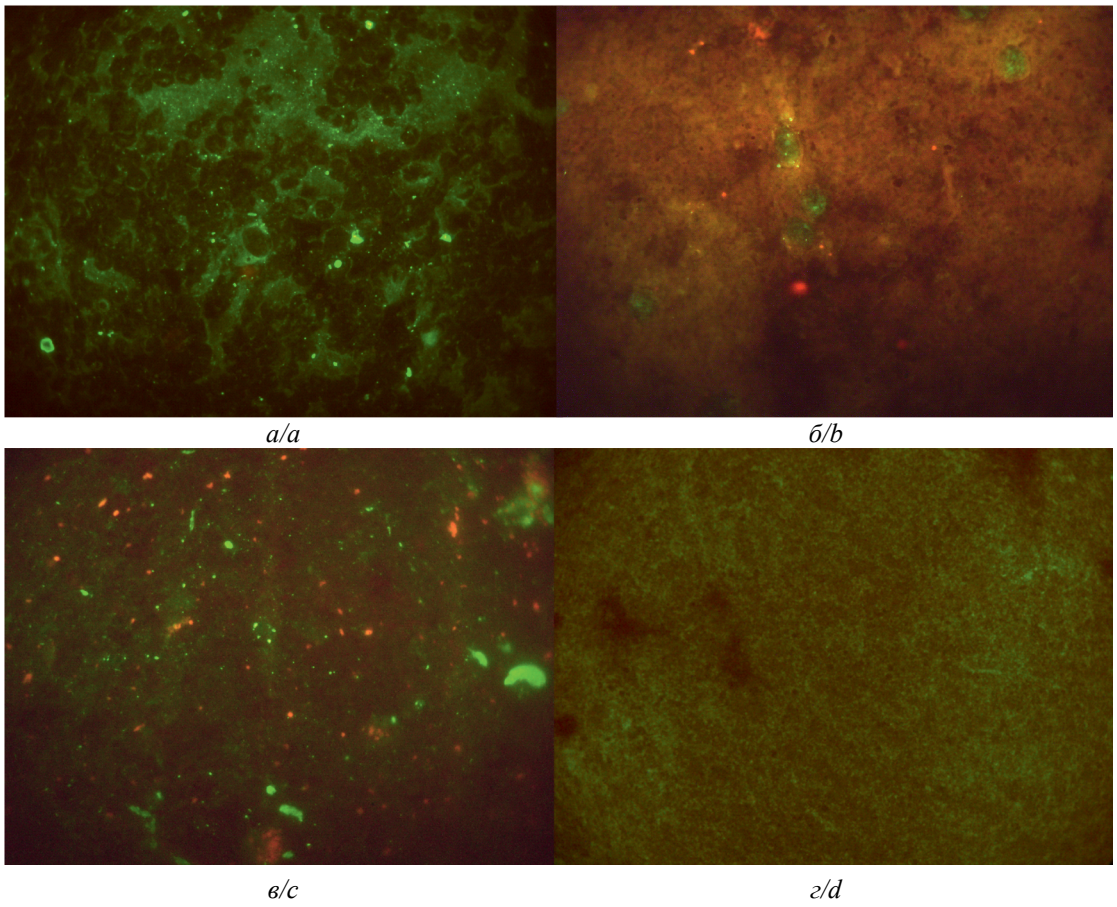

Fig. 2. Specific immunofluorescence of rabies lyssavirus antigen in brain imprints obtained during MFA using polyclonal Ig (FGBI "Federal Centre for Animal Health"). The prints were made from primary material (brain): *a* – human, Amur Region, 2019; *b* – human, Primorsky Territory, 2019; *c* – human, Primorsky Territory, 2021; *d* – healthy white mice. Olympus CX41 microscope, ×10 eyepiece, ×100 objective, DP 72 documentation system, oil immersion.

Рис. 2. Специфическая иммунофлуоресценция антигена лиссавирусов бешенства в отпечатках головного мозга, полученная методом флуоресцирующих антител с использованием поликлонального Ig (ФГБУ «ВНИИЗЖ»). Отпечатки сделаны с первичного материала (головной мозг): *а* – человек, Амурская область, 2019 г.; *б* – человек, Приморский край, 2019 г.; *в* – человек, Приморский край, 2021 г.; *г* – здоровые белые мыши. Микроскоп Olympus CX41, окуляр ×10, объектив ×100, система документирования DP 72, масляная иммерсия.

mary material was 4.3 lg $LD50_{0.03}$ and 5.4 lg $LD50_{0.03}$ at the 4th passage (**Table 2**).

Amplification and sequencing of Rus(Amur)8947H_2019, Rus(Primorsky)8949H_2019 and Rus(Primorsky) 9220H_2021 isolates yielded the fragments of nucleoprotein gene sequences, the length of which was 1258 nt (accession numbers in GenBank: OQ377548 – OQ377550).

Using the BLASTN 2.12.0+ program, we have found that sequences of Rus(Amur)_8947H_2019, Rus(Primorsky)_8949H_2019, and Rus(Primorsky)_9220H_2021 viruses were identical to those of Irkut lyssaviruses: the Ozernoe virus isolated from the individual who died in the Primorsky Territory in 2007 had 98.17–99.68% identity; the IRKV-THChina12 virus isolated in 2012 from a bat in China (Tonghua, Jilin province) had 98.17–98.97% identity; the FX17 virus isolated in 2017 from a dog in China (Fuxin) had 98.01–98.81% identity; the Irkut virus isolated from a bat in Irkutsk in 2002 had 92.75–93.15% identity (**Table 3**).

The similarity with the Hamburg lyssavirus (EBLV-1, isolate 13424, Spain, 1989) was 79.08–79.28% and with classical rabies lyssaviruses – 76.75–77.64% (**Table 3**).

It has been found that the viruses responsible for human deaths in the Amur Region and the Primorsky Territory form a distinct monophyletic cluster with lyssaviruses belonging to the Irkut species with 100% bootstrap support (**Fig. 3**).

Discussion

The described three deceased patients did not demonstrate any typical clinical picture of rabies (hydrophobia, aerophobia, photophobia), which would be indicative of the lyssavirus etiology of encephalitis. Considering the increasing severity of the disease and based on the clinical and epidemiological data, one patient was diagnosed with unspecified viral encephalitis, while for two other cases, the epidemiological anamnesis served as the basis for diagnosing encephalitis of lyssavirus etiology.

Table 2. Characteristics of the isolated lyssaviruses that caused the death of people after being bitten by bats
Таблица 2. Характеристика выделенных лиссавирусов, вызвавших гибель людей после укусов рукокрылыми

Characteristics Характеристика	Rus(Amur)8947H_2019	Rus(Primorsky)8949H_2019	Rus(Primorsky) 9220H_2021
Incubation period at 1 infection Инкубационный период при 1 заражении	10–13 days (mean 11.5) 10–13 дней (в среднем 11,5)	7–10 days (mean 8.6) 7–10 дней (в среднем 8,6)	6–8 days (mean 7.1) 6–8 дней (в среднем 7,1)
Incubation period at passage 3 Инкубационный период на 3-м пассаже	5 days 5 дней	5 дней 5 days	4-5 дней 4-5 days
Incubation period at passage 5 Инкубационный период на 5-м пассаже	4–5 days 4–5 дней	4–5 дней 4–5 days	4–6 дней 4–6 days
Virus titer in primary material Титр вируса в первичном материале	4,4 lg $LD50_{0,03}$	4,6 lg $LD50_{0,03}$	4,3 lg $LD50_{0,03}$
Virus titer at the level Титр вируса на уровне	4-го пассажа passage 4 4,7 lg $LD50_{0,03}$	6-го пассажа passage 6 5,6 lg $LD50_{0,03}$	4-го пассажа passage 4 5,4 lg $LD50_{0,03}$

Table 3. Search for nucleotide identity of obtained sequences using BLASTN 2.12.0+
Таблица 3. Поиск идентичности нуклеотидов полученных сиквенсов с помощью BLASTN 2.12.0+

Viruses Вирусы		Identity of nucleoprotein genome fragments (1258 n.p., position 71-1328 relative to RefSeq NC_001542.1), % Доля идентичности фрагментов генома нуклеопротеина (1258 н.о., позиция 71-1328 относительно RefSeq NC_001542.1), %		
Irkut lyssaviruses (IRKV) Лиссавирусы Иркут (IRKV)		Rus(Amur)_8947H_2019	Rus(Primorsky)_8949H_2019	Rus(Primorsky)_9220H_2021
1	Ozernoe, 2007	98,17	99,68	99,13
2	THChina12, 2012	98,17	98,89	98,97
3	FX17, 2017	98,01	98,73	98,81
4	Irkut (NCBI RefSeq), 2002	93,15	92,83	92,75
Lyssavirus hamburg (EBLV-1, isolate 13424, Spain, 1989) (EBLV-1, изолят 13424, Испания, 1989 г.)		79,16	79,08	79,28
Лиссавирусы бешенства (RABV)		77,24–76,75	77,64–77,24	77,53–77,04

The fatal case with distinct clinical manifestations was reported in Zavitinsk in the Amur Region in 2020. A 45-year-old woman died after being bitten by a dog. The case was not laboratory confirmed as the material was collected improperly. However, pronounced hydrophobia, aerophobia, photophobia, hyperthermia, hypersalivation, aggressiveness, the fact of a dog bite made it possible to conclude that the woman's death was caused by rabies based on the clinical and epidemiological data.

Note that a contact with chiropters may not be noticed by an individual; therefore, the epidemiological anamnesis will not have any data regarding this contact. The infection caused by lyssaviruses that are different from classical rabies can develop without typical clinical signs. There are reports mentioning atypical symptoms of human rabies caused by chiropter bites (The World Health Organization, 2018), thus making it difficult to diagnose the disease. Therefore, if a patient's death is caused by acute encephalomyelitis of unknown etiology within 10–15 days from the onset of the disease, the autopsy material must be tested for lyssavirus infection. In the Sanitary Rules and Regulations SanPiN 3.3686-21, in the Sanitary and Epidemiological Requirements for Prevention of Infectious Diseases (section XXII), we updated the rules for rabies prevention and emphasized the significance of the above tests.

The south of the Far East is known as a natural focal region [40] where rabies lyssaviruses circulate in populations of foxes and racoon dogs. From time to time, wolves become involved in the epizootic process. During different periods of epizootic outbreaks, 40–60% cases of rabies are reported among domestic dogs [20, 21].

Over the last 20 years, in the Far East a total of 11 human deaths caused by encephalitis of lyssavirus etiology have been reported. In 3 cases, the sources of the rabies lyssavirus were domestic dogs and in 4 cases – wild carnivores (a fox – 3 cases, a wolf – 1 case) [20]. Chiropters were the source of the Irkut lyssavirus in other 4 cases [28], including the cases described in this article.

The Irkut lyssavirus was first isolated from a greater tube-nosed bat (*Murina leucogaster*) in Irkutsk in 2002 [23]. In 2007, a human death caused by the above lyssavirus was reported in Ozernoe village in the Primorsky Territory [28] (**Fig. 4**).

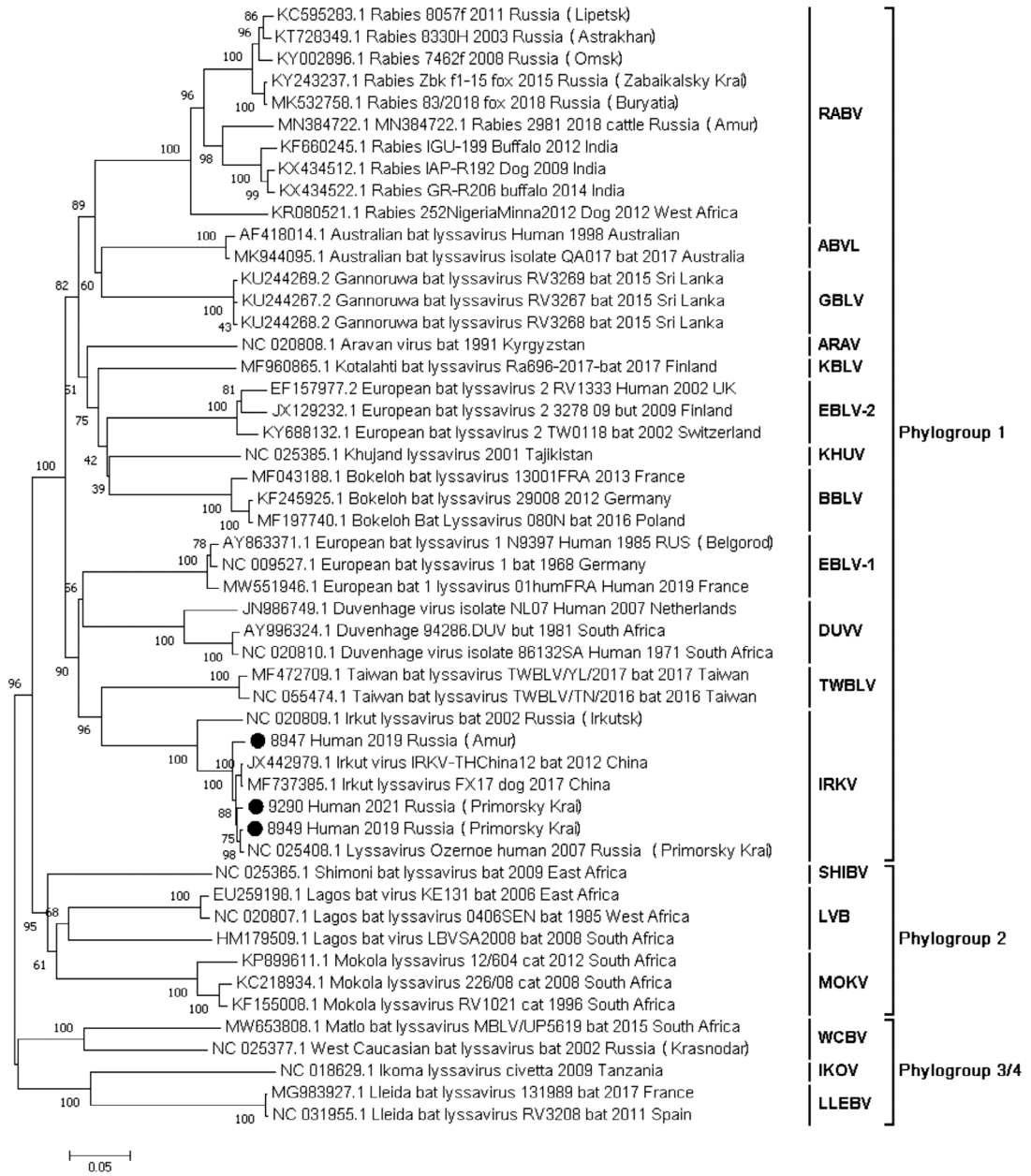

Fig. 3. Phylogenetic dendrogram obtained by the Neighbor-Joining method for 51 isolates of lyssaviruses of known species based on the alignment of the nucleoprotein gene sequences (*N*, 1258 bp). The nodes indicate the percentage of duplicate trees (bootstrap support, %) in which associated taxa are grouped together in the bootstrap test (1000 repetitions). The values of the bootstrap support indicators reflect the stability of the topology of the dendrogram and are significant at values > 70%. The analysis was performed in MEGA7 software.

Рис. 3. Филогенетическая дендрограмма, полученная методом Neighbor-Joining для 51-го изолята лиссавирусов известных видов на основании выравнивания последовательностей гена нуклеопротеина (*N*, 1258 н.о.). В узлах указана доля дублирующих деревьев (бутстреп-поддержка, %), в которых ассоциированные таксоны сгруппированы вместе, в бутстреп-тесте (1000 повторов). Значения показателей бутстреп-поддержки отражают устойчивость топологии дендрограммы и достоверны при значениях > 70%. Анализ выполнен в MEGA7.

The above virus has been detected not only in Russia. In 2012, the Irkut lyssavirus was isolated from a greater tube-nosed bat in Jilin province (Jilin) in the center of Northeast China [38]. The repeated discovery of the pathogen in the same species of chiropters can be indicative of its species specificity. A few years later, in 2017, in the Fuxin County in China, the Irkut virus was isolated from a dead domestic dog that had bitten a human [41]. It was the first evidence of possible transmission of the Irkut bat virus to terrestrial carnivores and from them – to humans.

The identity analysis with the BLASTN program and the phylogenetic analysis have demonstrated that the obtained sequences of the Rus(Amur)_8947H_2019, Rus(Primorsky)8949H_2019, and Rus(Primorsky)_9220H_2021 viruses isolated in 2019 and 2021 are clustered with IRKV lyssaviruses and are identical to repre-

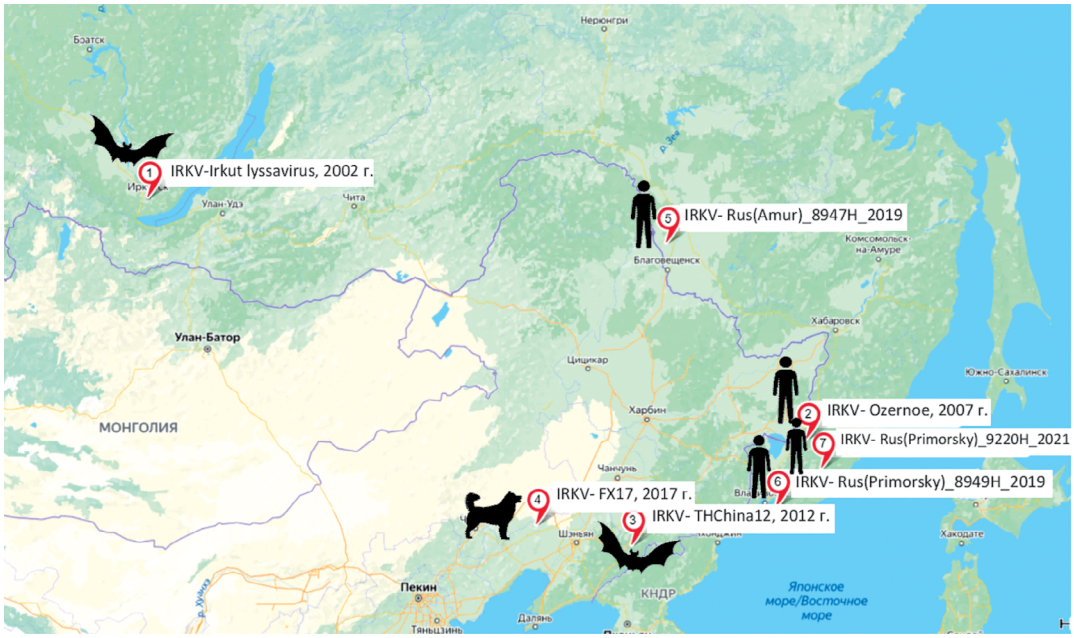

Fig. 4. Cases of detection of Irkut lyssavirus (IRKV) in chronological order: 2002 – Russia, Irkutsk, Bat; 2007 – Russia, Primorsky Krai, Yakovlevsky district, Ozernoye village, human; 2012 – China, Jilin province, Bat; 2017 – China, Fuxin County, domestic dog; 2019 – Russia, Amur Region, Blagoveshchensk, human; 2019 – Russia, Primorsky Territory, Fokino, human; 2021 – Russia, Primorsky Krai, Chuguevsky district, Zavetnoe village, human.

Рис. 4. Случаи выявления лиссавируса Иркут (IRKV) в хронологическом порядке: 2002 – Россия, Иркутск, большой трубконос; 2007 – Россия, Приморский край, Яковлевский район, с. Озёрное, человек; 2012 – Китай, провинция Гирин, большой трубконос; 2017 – Китай, округ Фусинь, домашняя собака; 2019 – Россия, Благовещенск, Амурская область, человек; 2019 – Россия, Приморский край, ГО ЗАТО Фокино, человек; 2021 – Россия, Приморский край, Чугуевский район, с. Заветное, человек.

sentatives of the species by more than 90%. It is known that the identity threshold for *N* gene nucleotide sequences, which is responsible for delineation of all lyssaviruses into species, is 82% [42]. The similarity with other species (EBLV-1 and RABV) ranged from 79.28 to 76.75%, thus demonstrating that the detected viruses belonged to the IRKV species.

The analysis of phylogenetic relationships, which was performed with the MEGA7 software for sequences of the *N* gene fragment in all the known species of lyssaviruses from GenBank (1258 nt, $n = 51$), showed that the viruses that caused human deaths in the Amur Region and the Primorsky Territory form a distinct monophyletic cluster with Irkut lyssaviruses with 100% bootstrap support (**Fig. 3**).

All the IRKV viruses, though they were found in relatively distant geographical locations, are closely related (**Fig. 3**, **4**). There were at least three human deaths caused by rabies transmitted by bats in Northeast China: in the Tonghua County in the 1990s and in 2002, and in the city of Longjing in 2010. These cases were diagnosed only clinically, without laboratory confirmation. The species of chiropters responsible for the human deaths were not identified. Reports about incidents of bat attacking humans were rare [38]. Earlier, in Asia, a few non-characterized cases of lyssavirus infection in chiropters were reported [11]. In 1967, in Thailand, lyssavirus antigens were detected in two Malayan short-nosed fruit bats (*Cynopterus brachyotis*) using the fluorescent antibody test [43]. In 1978, the lyssavirus was detected in the grey-headed flying fox (*Pteropus poliocephalus*) in India [44]. These data imply the possibility of wider distribution of the Irkut lyssavirus. It can have wide distribution across the geographic range of the greater tube-nosed bat, including Siberia and the Far East. Other species of chiropters can be involved in the circulation of the pathogen.

At this stage, we have shown the benefits of using molecular and genetic methods for detection of representatives of the genus *Lyssavirus*, which do not belong to the classical rabies lyssavirus; we have pointed out the significance of their application for interpreting human fatal cases associated with encephalitis of unclear etiology and assessed diagnostic capabilities of PCR test-systems from Russian manufacturers. The successful detection of non-classical rabies lyssaviruses using a Russian diagnostic kit was an exceptional example, as all the available Russian PCR test-systems are designed specifically for detection of the classical rabies lyssavirus.

For example, the PCR test-system from Syntol, which was used for diagnosis, was designed for detection of the specific RNA of the classical rabies lyssavirus by detection of two genome regions: the region encoding the nucleoprotein (*N*) gene and the region encoding the gene of RNA-dependent RNA polymerase (*L*). In this assay, all classical rabies viruses were detected by the channel identifying nucleoprotein fragments. The specific fragments of lyssavirus RNA from the patients who died following bat bites were identified only by

the *L* gene detection channel, while the nucleoprotein region was not detected by this test system. Therefore, we assumed that the Syntol test system detected another lyssavirus, which did not belong to the classical rabies lyssavirus and possibly belonged to phylogroup I (see below). At the same time, another assay designed for detection of the nucleoprotein region of the classical rabies virus common in Russia did not detect the above viruses and demonstrated the absence of its ability to identify the above pathogen.

During the virological study, viruses were isolated using the white outbred mouse bioassay; their specificity was confirmed by the fluorescent antibody test with polyclonal anti-rabies immunoglobulin. The test was successful, as the *N* gene is highly conserved; it induces formation of cross-reacting and complement fixing antibodies, while lyssaviruses of different species demonstrate broad antigenic cross-activity at the level of detection of the N protein antigen [3]. Therefore, standard diagnostic techniques designed for detection of the nucleoprotein (for example, the fluorescent antibody test), through reaction with the polyclonal anti-rabies immunoglobulin, can be used for detection of all lyssaviruses without species differentiation, using the same diagnostic kits.

Based on genetic distances and serological cross-activity, lyssaviruses are divided at least into three phylogroups. Phylogroup I is comprised of RABV, DUVV, EBLV-1, EBLV-2, ABLV, ARAV, KHUV, IRKV, BBLV, GBLV, TWBLV, KBLV. Phylogroup 2 includes LBV, MOKV, SHIBV. The other species – WCBV, IKOV, and LLEBV – cannot be included in any of these phylogroups. Each phylogroup is characterized by relative conservation of glycoprotein antigens inducing the production of virus-neutralizing antibodies responsible for antiviral immunity (the identity of amino acids in the ectodomain is >74%). Cross-neutralization between lyssaviruses belonging to different phylogroups is absent (the identity of amino acids in the ectodomain is <62%). As a result, commercially available vaccines and immunoglobulins against rabies, which are based on strains of the classical virus, generally induce immune protection against lyssaviruses of phylogroup I rather than other lyssaviruses [7].

The Irkut lyssavirus belongs to phylogroup I [7]; therefore, the existing anti-rabies products should provide protection against it. However, the experimental findings of American and Chinese studies have shown that commercial biopharmaceuticals against rabies do not provide 100% protection against the infection caused by IRKV, which would be as reliable as the protection against infection with the classical rabies lyssavirus [45–47].

Conclusion

1. During 1977–2021, seven human deaths from bat bites were reported in Russia: two deaths were reported in the Lugansk People's Republic, Lugansk (Voroshilovgrad) and Molodogvardeisk; one death – in the Belgorod Region, Belgorod; one death – in the Amur Region, Blagoveshchensk; three deaths – in the Primorsky Territory, Ozernoe village, Fokino, Zavetnoe village.

2. Out of four species of lyssaviruses circulating in Russia – *Lyssavirus rabies*, *Lyssavirus hamburg*, *Lyssavirus irkut*, *Lyssavirus caucasicus* – representatives of the first three species caused human deaths; the last three species were isolated from bats.

3. The reported human deaths caused by lyssavirus infection in the south of the Far East Region imply that the role of chiropters in epidemiology of this infection was underestimated in Russia and suggest the possibility of under-diagnosis of lyssavirus infection after contacts with these animals.

4. In Russia, bat populations must be monitored for infection with lyssaviruses, taking into consideration that the Irkut lyssavirus has been detected in the greater tube-nosed bat in Russia and China, thus implying the possible species specificity of the pathogen.

5. The epidemiological and public health authorities must be aware of the possibility of chiropters to transmit lyssaviruses. With a high species diversity of bats and rapid emergence of new species of lyssaviruses, none of the Russian regions can be considered free of these pathogens.

6. Considering the difficulty of clinical diagnostics of the etiology of encephalitis caused by lyssaviruses that are different from the classical rabies lyssavirus, the autopsy material from the patients who died from encephalomyelitis of unknown etiology must be tested for the presence of the lyssavirus pathogen within 10–15 days from the onset of the disease.

7. The Russian commercial diagnostic kits for the fluorescent antibody test are efficient for detection of the Irkut lyssavirus antigen. The Russian PCR-based test-system from Syntol can be used for detection of the Irkut lyssavirus, but there is need for diagnostic kits that employ genus-specific primers. Molecular and biological methods offer good prospects for improvement of rabies diagnosis and further improvement of epidemiological surveillance and effectiveness of the system of biological protection of the population in Russia.

8. The fact that the identified pathogens belong to the Irkut virus implies that the Russian vaccines available on the market provide effective protection against this pathogen. Nevertheless, the evaluation of the existing immunobiological products in terms of their effect on the Irkut virus and the development of new products remain priority tasks.

9. Summarized data on detection of the Irkut lyssavirus and the proven facts of its transmission to dog populations increase the urgency of vaccination of domestic animals to prevent any risk of their infection with this pathogen.

10. There is an urgent need for improvement of rabies awareness and educational campaigns to prevent contacts of the population with rabid animals, including bats, as well as for better encouragement of timely seeking medical attention if bitten by animals. The injuries caused by chiropters must be reported and documented.

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