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Serological and molecular investigations of Orthohantaviruses in the Republic of Kazakhstan

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Key Words

Orthohantavirus, Tula virus, Puumala virus, serology, fever of unknown origin, Hemorrhagic fever with renal syndrome, Republic of Kazakhstan, rodent, one health

Abstract

Background: Orthohantaviruses are zoonotic pathogens that play a significant role in public health. Several small mammals are reservoirs of orthohantaviruses and can cause hemorrhagic fever with renal syndrome in humans in Eurasia and hantavirus cardiopulmonary syndrome in the Americas. Kazakhstan is a Central Asian country with a vast territory and several zoonotic diseases. West Kazakhstan region is an officially endemic region for orthohantaviruses with officially registered human cases and antigen findings in natural host reservoirs. However, there was never an initiative to undertake molecular-biological and serological analyses in humans or in host reservoirs in endemic and non-endemic regions. In this thesis, I demonstrate serological and molecular-biological studies in humans and small mammals in different areas of Kazakhstan.

Methods: Patients with fever of unknown origin and patients with suspected cases of hemorrhagic fever with renal syndrome are included in a serological and molecular-biological study in different endemic and non-endemic areas of Kazakhstan. In parallel, natural host reservoirs are investigated using molecular-biological methods.

Results: In total 802 patients are included in a study with fever of unknown origin in Almaty and Kyzylorda regions. A serology screening showed IgG antibodies in 22.2% and for IgM in 0.5% of the cases by ELISA. Further testing of positive samples by immunoblot and immunofluorescence assay showed that the genotypes Puumala, Hantaan, and Dobrava were the main drivers of an infection. In a second study, 139 patients with suspected cases of haemorrhagic fever with renal syndrome from West Kazakhstan and Almaty city showed an IgG seropositivity of 23.7% and an IgM seropositivity of 5%. Here, immunoblot testing of positive samples showed the Puumala serotype in IgM positive samples and this was confirmed by sequencing. In a third study, 15 out of 621 small mammals captured were positive for orthohantavirus, one sample from West Kazakhstan and 14 samples from Almaty region. Positive samples were found in two species of rodents, namely *Microtus arvalis* (13/15) and *Dryomys nitedula* (2/15). By sequencing parts of S and L segments the occurrence of Tula virus in these two regions could be confirmed.

Conclusion: Our results show that orthonataviruses exist in so far non-endemic regions of Kazakhstan. Hence, it is important to establish contemporary laboratory diagnostic tools for the investigation of orthohantaviruses in humans and natural host reservoirs in all regions of Kazakhstan in order to clarify true endemicity.

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List of abbreviations

BSL	Biosafety level
DNA	Deoxyribonucleic acid
DOBV	Dobrava-Belgrade virus
ELISA	Enzyme-linked immunosorbent assay
FRNT	Focus-reduction neutralisation test
FUO	Fever of unknown origin
HNTV	Hantaan virus
IFA	Immunofluorescence assay
PCR	Polymerase chain reaction
PUUV	Puumala virus
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAAV	Saaremaa
SEOV	Seoul virus
TULV	Tula virus
WHO	World Health Organization

List of publications

A. Tukhanova, N., Shin, A., Abdiyeva, K., Turebekov, N., Yeraliyeva, L., Yegemberdiyeva, R., Shapiyeva, Zh., Froeschl, G., Hoelscher, M., Wagner, E., Rösel, K., Zhalmagambetova, A., Musralina, L., Frey S., Essbauer, S. (2020). Serological investigation of orthohantaviruses in patients with fever of unknown origin in Kazakhstan. Zoonoses and Public Health, 67: 271–279.

B. Tukhanova, N., Shin, A., Turebekov, N., Nurmakhanov, T., Abdiyeva, K., Shevtsov, A., Yerubaev, T., Tokmurziyeva, G., Berdibekov, A., Sutyagin V., Maikanov, N., Zakharov, A., Lezdinsh, I., Yeraliyeva L., Froeschl, G., Hoelscher M., Frey, S., Wagner, E., Peintner, L., Essbauer S. (2022). Molecular characterisation and phylogeny of Tula virus in Kazakhstan. Viruses. 16(6), 1258

1. My contribution to the publications

1.1 Contribution to paper A

The paper A “Serological investigation of orthohantaviruses in patients with fever of unknown origin in Kazakhstan” was published in January 2020. I am the first author and contributed serological investigation (ELISA, IFA, Immunoblot, RT-PCR) of samples. After the methodological analyses, I evaluated the results of the serological study, interpreted the data, and analysed the entire data gathering process. I wrote the original draft of the manuscript, and supported the corresponding author with reviewing and editing the final version of the manuscript. All listed processes were under the supervision of all my supervisors.

1.2 Contribution to paper B

The paper B “Molecular characterisation and phylogeny of Tula virus in Kazakhstan” was published in June 2022. As the first author I contributed to the conceptualization, the study design, data collection, capturing of rodents, necropsy and transporting of rodent samples, tissue homogenization and RNA extraction of samples under BSL-3 condition. I was responsible for the conduction of the molecular-biological investigation (real-time RT-PCR, RT-PCR), the preparation and shipment of samples for sequencing, the coordination with the sequencing facility and subsequent sequencing data analysis and full data analysis. I took responsibility of writing the original draft, responding to the reviewers’ comments and reviewing and editing final version of the manuscript. All those steps were under the supervision of all supervisors and in coordination with the project team.

2. Introductory summary

2.1 Background

The genus of *Orthohantavirus* (family *Hantaviridae*) is geographically widely distributed and presents a significant impact on public health [Vaheri et.al., 2015]. Many species of small rodents are natural host reservoirs of orthohantaviruses, meanwhile the virus is also detected in shrews, moles and bats [Holmes et.al., 2015, Laenen et.al., 2019].

Viruses of the family *Hantaviridae* have spherical or oval virions with a diameter of 80-120 nm. The genome consists of a single-stranded negative polarity RNA and divided in three segments. The large (L) segment encodes the viral RNA-dependent RNA polymerase (RdRp), the medium (M) segment encodes the glycoprotein precursor GPC, which is processed to the glycoproteins Gn and Gc and the small (S) segment encodes the nucleocapsid protein [Plyusnin 2002].

Rodent-borne orthohantaviruses can cause two distinct forms of disease in humans: I) hemorrhagic fever with renal syndrome (HFRS) in Eurasia and II) hantavirus cardiopulmonary syndrome (HCPS) that is mostly observed in the Americas. HFRS is a febrile illness that begins with flu-like symptoms and may progress into shock, renal failure and hemorrhagic syndrome. HCPS is also a febrile illness but characterized by a respiratory failure with diffuse interstitial edemas [Akram et al., 2021].

In Eurasia HFRS caused by several orthohantaviruses species such as Hantaan virus (HNTV), Dobrava-Belgrade virus (DOBV), Seoul virus (SEOV), Puumala virus (PUUV), and Tula virus (TULV) [Bi et.al., 2008, Avšič-Županc et.al., 2019]. While orthohantaviruses are asymptomatic in their rodent reservoir humans - as a dead end host - often develop severe symptoms. Persistently infected rodents constantly shed the virus through their excreta. Humans get infected by inhalation of aerosolized contaminated excreta of infected rodents and rarely also by rodent bites [Kruger et.al., 2015]. Human to human transmission is only very seldom reported. Only the Andes virus causing HCPS was described in Argentina and Chile to establish a man-made transmission line [Chaparro et.al., 1998, Padula et.al., 1998, Alonso et.al., 2020].

Each orthohantavirus is carried by a distinct rodent species or closely related species evolving in a long-standing virus-host relation [Avšič-Županc et.al., 2019]. Hence, the distribution of different Orthohantavirus-species is linked to the distribution of the host species. For instance, PUUV is carried by *Myodes glareolus*, a rodent that is very dispersed in many European countries. TULV is carried by *Microtus arvalis* and by some other *Microtus* species and can be mostly found in Central and Eastern Europe and in Asia. DOBV can be subdivided into four genotypes, the Dobrava, Kurkino, Saaremaa and Sochi virus. All of them are transmitted by several *Apodemus* species such as *A. flavicollis, A*. *agrarius* or *A.ponticus* and is a relevant infection threat in southeastern Europe, north and central Europe and the southern part of Russia [Klempa et.al., 2013, Chen et.al., 2019, Vaheri et.al., 2021]. SEOV successfully spread worldwide, as it is carried by *Rattus norvegicus* and *R. rattus*, two rodent species that follow global transportation routes. Last, HNTV is carried by *Apodemus agrarius* and can be found in Asia [Zhang et.al., 2007, Zou et.al., 2016, He et.al., 2019].

In European countries the main causative agent of HFRS in humans are PUUV and DOBV, whereas HNTV virus is the main driver of HFRS in Asia [Vaheri et.al., 2015, Zou et.al., 2016, Avšič-Županc et.al., 2019]. In Europe, the clinical picture of HFRS was further subdivided. Especially PUUV is known to cause Nephropathia epidemica (NE) a mild form of HFRS with case fatality rates of 0.08-0.4%. Classical HFRS is reported to be caused by other European Orthohantavisues, however in various degrees of seriousness. DOBV can cause moderate to severe forms of HFRS with case fatality rates up to 9-12% [Essbauer et.al., 2006, Heyman et.al., 2009, Avšič-Županc et.al., 2019]. Further, SEOV cause moderate form of HFRS with a case fatality rate of 1%, single cases also reported Seoul associated HFRS in Europe [Jameson et.al., 2013, He et.al., 2019]. HNTV causes the severest form of HFRS in Asian countries and Far East Russia with case fatality rates up to 15% [Jonsson et.al., 2010, Kariwa et.al., 2012, Zou et.al., 2016]. NE/HFRS cases are strongly associated to their natural harbour of carrier rodents by natural and occupational factors [Krautkramer et.al., 2013, Singh et.al., 2021]. The pathogenicity of TULV in humans is still ill-defined. Only few cases of HFRS induced by TULV have been recorded in Europe. Most infections with TULV remain undiagnosed due to the usual absence of severe symptoms or organ failures [Schultze et.al., 2002, Klempa et.al., 2003, Zelena et.al.,2013, Hofmann et.al., 2021].

The epidemiological pattern and the infection rates among humans are dependent on the host reservoirs and has particular periodic characteristics. Those infection fluctuations are influenced by rodent reproduction dynamics, where climate conditions and food supply may affect population cycles. Long-term observations showed that the dynamics of the incidence of HFRS in Europe is characterized by rises every 3-4 years, due to the periodicity of the local rodent population. Human cases can be observed in two seasonal peaks, one in summer-autumn when urban citizens get infected in their summer vacations and the autumn-winter period when the density of rodents rises and they start to migrate to human dwellings where additional conditions for human infection arise [Faber et.al., 2019, Krautkrämer et.al., 2022].

The clinical picture of HFRS is variable depending on the serotype that causes the disease. The disease typically progresses through five phases, starting with the febrile phase, followed by the hypotensive shock-, oliguric-, polyuric-, and finally concluded by the convalescent phase. Some of these phases may overlap or remain absent. Infections can range from asymptomatic or mild forms or lead to acute renal failures and haemorrhagic manifestations [Jiang et.al., 2016, Avšič-Županc et.al., 2019]. Fever, headache, back/abdominal pain, nausea/vomiting are common clinical findings not specific for HFRS. The haemorrhagic complications in infected patients range from local haemorrhages to massive haemorrhages. Ocular findings are more common in the acute phase [Jonsson et.al., 2010, Jiang et.al., 2016, Krautkrämer et.al., 2022]. Acute renal failure can occur frequently in HFRS and may result in acute tubule-interstitial nephritis [Jiang et.al., 2016]. The laboratory findings may comprise leukocytosis, thrombocytopenia, elevated creatinine levels as well as proteinuria and haematuria [Jiang et.al., 2016, Avšič-Županc et.al., 2019, Singh et.al., 2021]. The diagnostic tools of HFRS are based on clinical findings (fever followed with abdominal pain, thrombocytopenia/haemorrhagic signs, and acute renal failure). The laboratory confirmation is usually performed by serology. An enzyme-linked immunosorbent assay (ELISA) on IgM and IgG against orthohantavirus antigens is the most common diagnostic tool for the diagnosis of HFRS. Further, immunoblot and immunofluorescence assays (IFA) are commonly

used for the diagnosis of HFRS. Molecular-biology methods in the form of a RT-PCR is rarely used in patients with HFRS. As the viremia is rather short and only lasts for about five to seven days it is difficult to isolate living virus from patients since they rarely report symptoms during this time [Vaheri et.al., 2013, Avšič-Županc et.al., 2019, Vaheri et.al., 2021]. However, traces of orthohantaviruses in the form of viral RNA remain detectable in patients' blood, serum, urine, cerebrospinal fluid, or saliva in an early stage of the disease, and therefore reverse transcriptase polymerase chain reaction (RT-PCR) is a reliable tool to identify a viral infection. Nevertheless, molecular-biological methods are mostly used for the investigation of host reservoirs and for monitoring and identifying the molecular epidemiology of orthohantaviruses in rodents but not for patient diagnostics [Avšič-Županc et.al., 2019, Krautkrämer et.al., 2022].

2.1.1 Orthohantavirus in Kazakhstan

Kazakhstan is a large Central Asian country (Figure 1) with a diverse landscape that includes forest-steppes, steppes, semi-deserts, desserts and mountain ranges [Atlas 2010]. In this wide range of geographic settings Kazakhstan developed several natural foci of important zoonotic pathogens such as *Yersinia pestis, Bacillus anthracis, Francisella tularensis, Leptospira, tick-borne encephalitis virus (TBEV), Crimean-Congo haemorrhagic fever virus (CCHFV)* and orthohantavirus [Atlas 2010, Abdiyeva et.al., 2019, Peintner et.al., 2021].

Figure 1: Map of Kazakhstan and Central Asia. Kazakhstan borders with the Russian Federation in the north and west, with China in the east, and with Kyrgyzstan, Uzbekistan, and Turkmenistan in the south.

The first human cases of HFRS were officially registered and described in the year 2000 in the West Kazakhstan region [Grazhdanov et.al., 2001, Zakharov et.al., 2010]. Since then, from 2000 to 2021, 248 cases of HFRS were officially registered and serological confirmed (by ELISA) in the

West Kazakhstan region [NCPHC, 2021]. Due to this first identification of human cases, an investigation of rodent host reservoirs was started. From 2001 to 2011 a total of 49,676 small mammals were screened for the presence of orthohantavirus antigen by ELISA (commercially distributed by the company Hantagnost, Russia) and a total of 1.53% of different species of small mammals were positive (*Myodes glareolus, Microtus arvalis, Apodemus uralensis,* and *Mus musculus*). Systematic monitoring demonstrated that four northern districts of the West Kazakhstan region (Borili, Bayterek, Shyngyrlau and Terekti) have natural endemic foci for orthohantaviruses [Bidashko et.al., 2004, Grazhdanov et.al., 2014]. However, investigations on orthohantaviruses in humans as well as in small mammals in West Kazakhstan using contemporary molecular methods were never applied.

The Dzhungarian Alatau mountain range of the Almaty region is located in the south-eastern part of Kazakhstan. This area has a vast array of different geographic zones such as lowland-foothills and low-mountain zones, mid-mountain forest-meadow-steppes and forest-meadow zones, high-mountain subalpine and alpine meadows and meadow-steppe zones and a high-mountain zone [Atlas, 2010, Sutyagin et al., 2010]. An investigation of small mammals in the Dzungarian Alatau mountain range lasting from 2010-2016 demonstrated that 2.2% of the rodents contain antigens of orthohantaviruses in *Microtus arvalis*, *Microtus oeconomus*, *Apodemus uralensis*, *Apodemus agrarius*, and *Mus musculus* species [Sutyagin et.al., 2017]. However, most of the reports did not specify the orthohantavirus on the species level. Only one report demonstrated the genomic sequences of TULV in tissue samples of *Microtus arvalis obscurus* in Almaty region (Taldykorgan and Karatal) [Plyusnina et.al., 2008]. Moreover, there are no human cases of HFRS officially registered in Almaty region so far.

2.2 Statement of the problem

In the Republic of Kazakhstan, the clinical manifestation of HFRS is often in-apparent or mild. Therefore, the real number of patients with HFRS are underestimated. Further, doctors often do not recognize mild forms of HFRS and appropriate diagnostics are not used or are not available in regional hospitals of Kazakhstan. In Kazakhstan, hospitals laboratory diagnostics only can be performed in areas that are officially endemic for a disease. In other areas with suspected and sporadic cases of this infectious disease no such diagnostic is supported. Only if an infection in a previously non-endemic area, combined with the proof of its occurrence in natural hosts, is proven by scientific methods, the public health legislation starts the process of enabling diagnostics at hospitals. However, the available diagnostic tools are rather rudimentary. No molecular biological methods were ever applied to learn more about the epidemiology of HFRS in patients in West Kazakhstan region. In addition, there is no molecular investigation of host reservoirs (Figure 2).

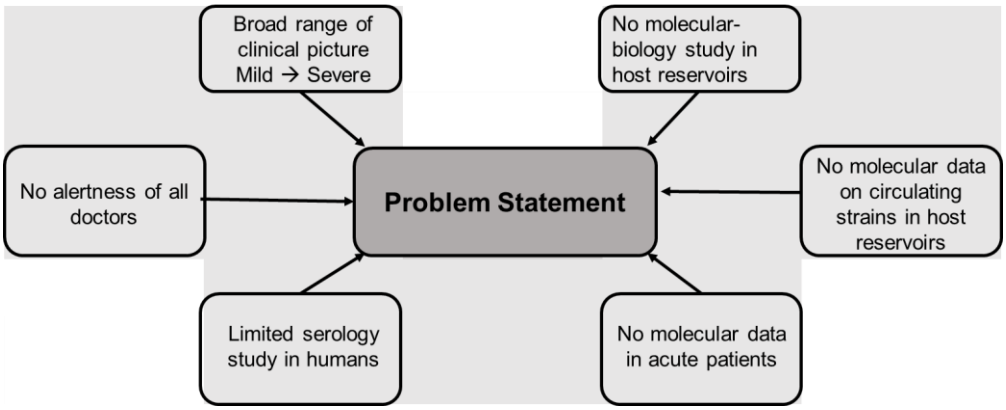

Figure 2: Problem statement on the situation of Orthohantaviruses in the Republic of Kazakhstan. In Kazakhstan, the endemic genus of Orthohantavirus is causing HFRS. However, exact information about the spread and impact of the virus is limited, since there is inefficient diagnostics, missing alertness of doctors, no longitudinal studies of rodents and no systematically recording of infected patients.

2.3 Objectives

Here I aim to initiate a systematic screening on the prevalence of orthohantavirus in the Republic of Kazakhstan. Orthohantaviruses reside naturally in rodents and may infect humans as a dead-end host. To draw a more detailed picture of the situation of orthohantavirus in Kazakhstan I intend to conduct three different studies in various oblasts (= regions) of the country:

1. Conduct a serological screening and differentiate the serotypes of orthohantaviruses in sera from patients with fever of unknown origin.
2. Estimate the prevalence of orthohantaviruses in rodents by molecular biological tests. All isolated viruses will undergo a molecular characterization to understand the species of the circulating strains of orthohantaviruses.
3. Serological and molecular-biological investigation of patients with suspected cases of HFRS.

2.4 Methods

To reach the main objectives I) a cross-sectional descriptive study was initiated and conducted to screen for orthohantavirus antibodies in serum samples of patients with fever of unknown origin (FUO) collected 2015-2016 in Almaty and Kyzylorda regions. II) To investigate host reservoirs of orthohantaviruses a molecular-biological study was performed to look for the prevalence of orthohantavirus in rodents. Subsequently a molecular biological characterization of circulating strains in West Kazakhstan and Almaty regions including Almaty city was conducted. III) In parallel, a human study to screen for orthohantavirus infections was applied among patients with suspected cases of HFRS in West Kazakhstan region and Almaty city. A summary of all research items is depicted in Figure 3.

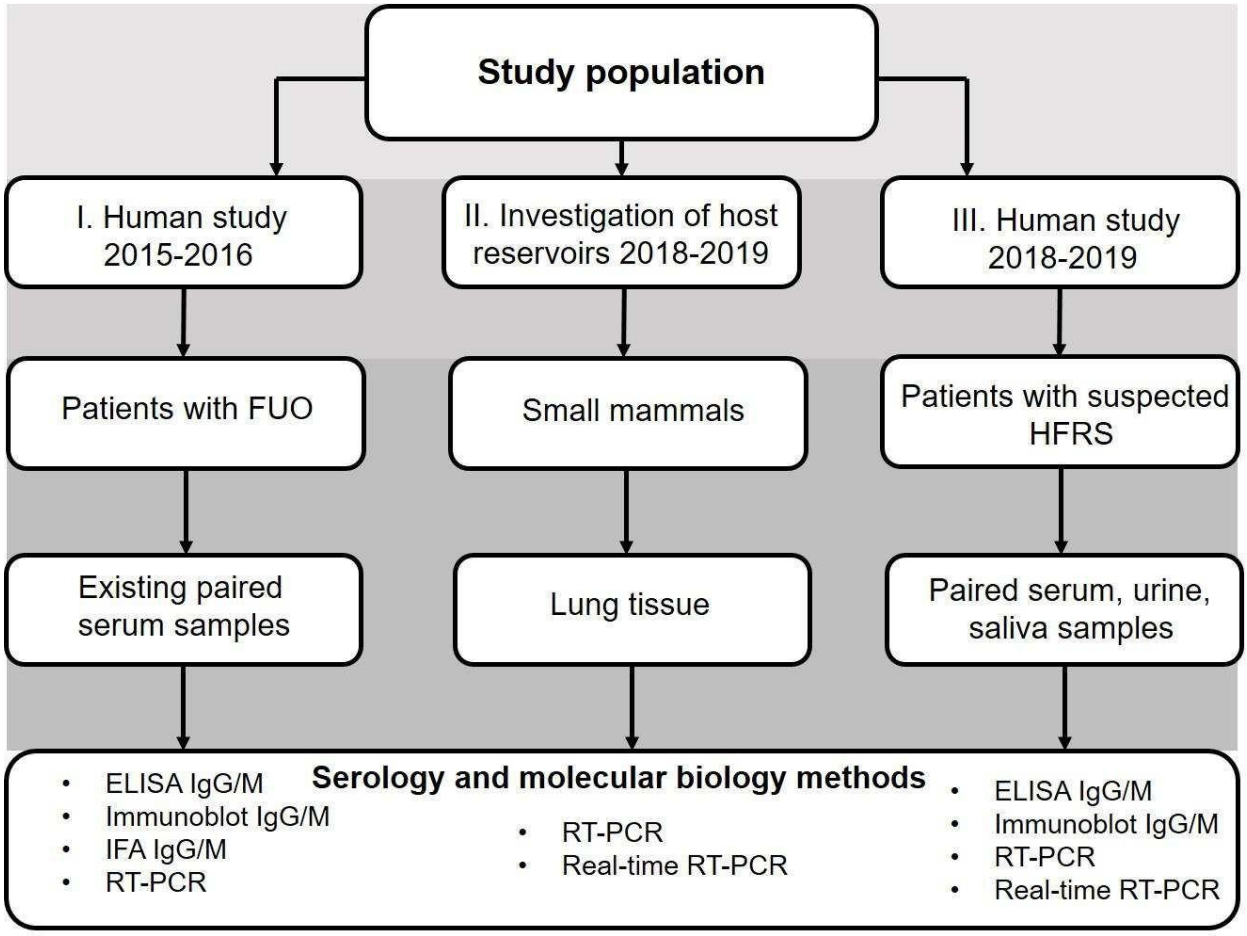

Figure 3: The employed method pipeline to investigate the spread of orthohantavirus in humans and rodents in Kazakhstan. Serology and molecular biology analysis is based on specimens from human samples (paired serum, saliva and urine) and tissue samples from small mammals.

2.4.1 Methods used in paper A

Patients with fever of unknown origin (FUO) (paper A) were recruited to the study in Almaty and Kyzylorda regions in 13 hospitals. A FUO was deformed as a sub-febrile and febrile temperature more than three days with unspecified cause for the fever. Included were both genders from an age ≥15 years old. Exclusion criteria were other laboratory confirmed diseases. A standardized

questionnaire was performed with the enrolled patients in a face-to-face interview. The questionnaire included 47 questions with modules on sociodemographic factors, living and housing conditions, contact to livestock, exposure to vector habitats and clinical symptoms. From each patient serum samples were collected at two time points, the first on the day of hospitalization (serum 1) and after 10-14 days (serum 2). These sera were analysed employing serological (ELISA IgG/M, Immunoblot IgG/M, IFA IgG/M) and molecular-biological (RT-PCR) methods using pan-Hanta primers.

2.4.2 Methods used in paper B

The investigation of host reservoirs (small mammals) for orthohantaviruses was conducted in 2018 - 2019 in West Kazakhstan and Almaty regions including Almaty city (paper B). The capturing of small mammal was performed at in total 30 sampling sites. In West Kazakhstan at 19 trapping sites, in Almaty region at four and in Almaty city at seven trapping sites in spring, summer, autumn and winter. Snap traps were set up overnight and in the early morning all captured small mammals were collected and transported on dry ice. After morphological identification by experienced zoologists, necropsy was performed and lung, kidney, liver, spleen, brain, heart, ears and transudate were collected aseptically and stored at -20°C until further processing that is homogenization and RNA extraction. Lung tissue samples were homogenized and RNA extracted according to manufacturer's instructions under BSL-3 conditions. RT-PCR and real-time RT-PCR were performed using pan-Hanta primers that amplify either the partial S or L segments. Positive PCR products were further purified and sequenced according to the manufacturer's instructions.

2.4.3 Methods used in human study

A separate study on patients with suspected cases of HFRS was conducted in 2018-2019 in parallel to the rodent study in West Kazakhstan region and Almaty city. In West Kazakhstan, samples were collected in West Kazakhstan regional infectious disease hospital and in Almaty city samples were collected in infectious disease hospital as well as in nephrology departments of the central hospitals. A suspected case of HFRS was defined by symptoms such as fever, backache, abdominal pain, thrombocytopenia or/and signs of haemorrhages or/and acute kidney failure. Both genders from the age ≥18 years old were included in the study. A paper-based face-to-face standardized questionnaire was further conducted to learn more about sociodemographic factors, conditions of living and housing, exposure to livestock and vector habitats and clinical symptoms. On the first day of hospitalization 1st serum, saliva and urine samples were collected and after 10-14 days a 2nd serum was collected. Serum samples were screened by ELISA for orthohanta IgG/M antibodies. Positive samples were further tested by Immunoblot IgG/M to identify the serotypes of orthohantaviruses. In the case of a positive IgM serum sample a parallel urine and saliva sample collection was initiated and those specimens were tested by molecular-biology methods (RT-PCR, real time RT-PCR) using pan-Hanta primers. Positive samples were further purified and sequenced.

2.4.4 Data analysis

In the descriptive analysis of the cross-sectional study (paper A) absolute numbers and percentage of sera positivity for IgG/M are presented. A Chi^2 test was performed for the estimation of the association between risk factors and seropositivity. P values of ≤ 0.05 were considered as statistically significant. Univariate analysis was conducted to calculate the odds ratio (OR) and the 95% confidence interval (CI) to identify possible risk factors.

In the investigation of host reservoirs (paper B) absolute numbers and percentages of positive small mammals are presented. Generated nucleotide sequences were aligned in BioEdit 7.2.5. for species identification the nucleotide sequences were blasted for similarity in the public database of the National Center for Biotechnology (www.ncbi.nlm.nih.gov/blast/). In detail, the sequences were assessed by the Basic Local Alignment Search Tool, using BLASTn and BLASTn optimized for highly similar sequences (MEGABLAST). Subsequently, phylogenetic trees were constructed in MEGA X and phylogenetic relationships among nucleotide sequences were reconstructed with the neighbour-joining (NJ) and maximum likelihood (ML) method based on the Tamura 3-parameter model.

2.4.5 Ethical Considerations

All included participants (patients with FUO and suspected cases of HFRS) signed an informed consent after an explanation of the objectives and methodology of the study by the researcher. To preserve participants' anonymity, we pseudonymized the data by developing participant identification numbers consisting of three digital numbers and the hospital identification number. The study protocol was approved by the Kazakhstan local ethics committee for human studies at the Kazakh National Medical University in Almaty, Kazakhstan (564–18) and the Ethics Committee of the Medical Faculty of the Ludwig-Maximilians-University Munich, Germany (18–631).

The rodent trapping was performed after ethical approval of Kazakhstan local ethics committee at National Scientific Center Especially Dangerous Infectious in Almaty, Kazakhstan (protocol #4, 08.01.18) and the Ethics Committee of the Medical Faculty of Ludwig-Maximilians-University Munich, Germany (18-631).

2.5 Results

2.5.1 Results of paper A (Cross-sectional study)

In total 950 patients with FUO presented in 13 hospitals Kyzylorda and Almaty regions. After cleaning for exclusion criteria, 802 patients were finally included in study, since they had paired serum samples and a completed questionnaire. A serological screening of the collected serum samples showed a reactivity for orthohantavirus IgG in 178/802 (22.2%) and for IgM in 4/802 (0.5%) of the samples by ELISA. In total 178 ELISA IgG-positive serum samples were further tested by immunoblot assay and IFA for the identification of the serotypes. Here the screenings showed a reactivity for PUUV, HTNV, and DOBV by immunoblot assay in 34/178 serum samples (19.1%). By employing the IgG IIFT method orthohantavirus species PUUV and DOBV were identified in 20/178 serum samples (11.2%, 5 positive in 1:10, 15 positive in 1:100 dilution). In total three of the four IgM positive ELISA serum samples were confirmed by immunoblot tests for the PUUV serotype. IFA showed in three serum samples a weak positive reactivity in 1:10 and 1:100 dilutions with SAAV, PUUV, DOBV, SEOV and HTNV serotypes. The four serum samples indicating an acute orthohantavirus infection originated from the Almaty region from three hospitals (Yessyk-2, Almaty-1 and Tekeli-1). All IgM-positive serum samples were additionally tested by RT-PCR to detect traces of RNA from orthohantaviruses. However, it was not possible to find orthohantavirus RNA in the patients' samples.

Of the four IgM positive patients, three were females of ages 22, 33 and 51 years and one male at the age of 19. Of the IgM-positive patients, two individuals lived in rural and two in urban areas. By correlating the infections with the patients daily activities it becomes apparent that half of the participants did garden and fieldwork before the first symptoms ($p = 0.864$) and three of them actually had seen rodents ($p = 0.213$). The clinical manifestations of the IgM positive subjects included fever (n = 4), headache (n = 3), weakness (n = 2), arthralgia (n = 2), back pain (n = 1), and nose congestion (n = 1).

To assess the potential risk factors for orthohantavirus infections, a univariate logistic regression was performed on in the ELISA IgG-positive serum samples. No significant association could be identified between risk factors such as sex, last trip into nature, house location in urban or rural area or the fact that the person had seen rodents with their status as an IgG positive person. Working in a garden and in the field, increased the risk of IgG seropositivity but it was not significant ($p = 0.05$). Furthermore, patients with an age ≤50 had 2.26 times more IgG seropositivity compared with the age higher than 50 years and this finding was statistically significant.

2.5.2 Results of paper B (Rodent study)

In total 621 small mammals were collected in West Kazakhstan (218), Almaty (199) regions and Almaty city (204), at alltogether 30 sampling points in 2018 and 2019. Collected small mammals represent 11 small rodent species including *Microtus arvalis* (86), *Myodes glareolus* (12), *Microtus kirgisorum* (49), *Apodemus ularensis* (259), *Mus musculus* (128), *Rattus norvegicus* (39), *Meriones meridianus* (2), *Dryomys nitedula* (15), *Sorex araneus* (1), *Sorex minutus* (2), and *Crocidura suaveolens* (28).

Out of all 621 collected small mammals 15 (2.4%) were positive for orthohantavirus, one sample from West Kazakhstan and 14 samples from Almaty region. No positive results were captured in Almaty city. The positive individuals represented two species of *Microtus arvalis* (n=13) and *Dryomys nitedula* (n=2). The molecular prevalence of positive individuals in *Microtus arvalis* was 15.1% (13/86) and in *Dryomys nitedula* (13.3%) 2/15 respectively. The age range of positive samples were adults (n=11) and sub-adults (n=4).

A partial sequence analysis of the S (346 nt) and L (184 nt) segments of the 15 positive samples showed the orthohantavirus species TULV in all the isolates. A sequence alignment of the sequences with S and L segments available from neighbouring regions was performed to understand the heritage of the sequences in a geographical perspective. The analysis of the S segment included 9 clades, from the Central North, Eastern North, Central South, Eastern South, Eastern Carpathian, Russia (Tula and Crimea) and Lithuania, Russia and China (Omsk, Xinjiang), Russia (Samara), West Kazakhstan and South-East Kazakhstan. Our results showed, that TULV from West Kazakhstan are in close evolutionary relationship with TULV described in Samara in the Russian Federation and thus is placed in one cluster. All Almaty region positive samples (Tekeli and Rudnichniy) have their own cluster and reside separated from all other TULV sequences isolated in Central Asia. Accordingly, the L segment results clustered in a similar pattern, however here only four different geographic locations could be created by published sequences, since for the L segment not so many data are publicly available. This resulted in a clustering of the 14 samples from Almaty region (Tekeli and Rudniychniy) shared with sequences from China (Xinjiang) and Turkey (Palandoken). The other sample from West Kazakhstan grouped its separate own cluster.

2.5.3 Results of serology and molecular biological analysis of suspected human cases of HFRS

In total 146 patients with suspected cases of HFRS from West Kazakhstan region (treated at one hospital) and Almaty city (treated at three hospitals) were recruited to this study. After excluding patients with incomplete recordings, 139 qualified for the final data analysis since for those patients paired serum, urine, saliva samples and completed questionnaire was available.

Figure 4: Regions where the suspected cases of HFRS patients were analysed and sequences were generated from IgM positives.

First, an ELISA based serology testing of serum samples was performed. This showed an IgG seropositivity in 23.7% (36/139) of the cases in the two examined regions, West Kazakhstan region (n=24/57, 42.1%) and Almaty city (n=12/82, 10.9%) respectively. Further testing of serum samples for IgM by ELISA showed an acute infection rate in 5% (7/139) of the cases, in West Kazakhstan region (n=5/57, 8.8%) and in Almaty city (n=2/82, 2.4%).

In a next step, the 36 IgG and 7 ELISA IgM-positive serum samples were tested by Immunoblot assay to identify the serotypes of the orthohantaviruses. These analyses showed a reactivity for PUUV, DOBV, and HNTV. The 36 ELISA IgG positive serum samples tested by Immunoblot showed positivity (strong to slight bands) for PUUV in nine samples, one slightly positive for HNTV and one slightly positive for DOBV. Hence, in total 11 from the 36 samples could be connected to an orthohanta species.

Similarly, among the seven ELISA IgM positive serum samples tested by Immunoblot IgM showed positivity for PUUV in six samples and reacted also with Sin Nombre virus. For the seventh sample, no differentiation by Immunoblot IgM was possible.

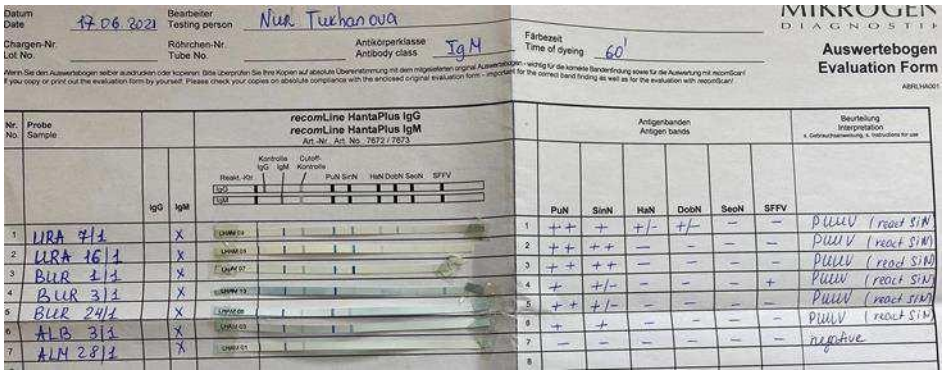

Figure 5: Incubated immunoblot strips to determine the species of the orthohantavirus with serum from IgM positive patients.

Since the commercially available immunoblot test kit is still prone to some impreciseness, all seven ELISA IgM positive serum samples, and their pairing urine and saliva was further tested by real-time RT-PCR to isolate and sequence genomic information from the acute infection in the patients. The sequencing analysis of the partial L (186 nt) segment showed the occurrence of PUUV in six samples of serum, saliva and urine from the IgM positive patients.

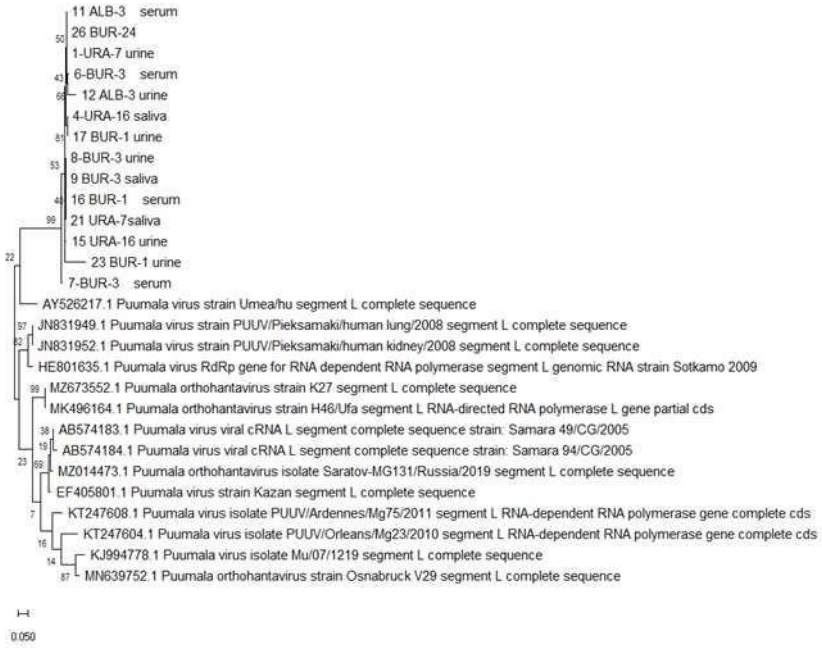

Figure 6: Phylogenetic analysis by the Maximum Likelihood method and a Tamura 3-parameter model of the partial L segment sequences (186 nt) of human samples of suspected cases of HFRS in Kazakhstan. The tree with the highest log likelihood (-1431.00) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 28 nucleotide sequences.

2.6 Discussion

Here we show – to our knowledge – the first time a complete molecular biological analysis of orthohantavirus infections in their natural hosts, in patients with fever of unknown origin (FUO) and in patients with suspected cases of HFRS in the Republic of Kazakhstan.

The clinical signs of the majority of zoonotic pathogens present in Kazakhstan are non-specific. The symptoms and differential diagnosis of the diseases are similar and differentiation is impossible without a laboratory confirmation. Furthermore, a limitation of available diagnostic tools for different zoonotic pathogens in different areas of Kazakhstan may result in the misinterpretation of febrile diseases. Here we demonstrate that orthohantaviruses are one cause for fever of unknown origin in Kazakhstan, far beyond the currently official endemic regions.

Human studies as well as studies on host reservoirs are routinely performed in many countries where orthohantaviruses are endemic [Bi et.al., 2008, Hukic et.al., 2010, Kruger et.al., 2015], often in longitudinal multi-year bio-surveillance. Seroprevalence studies for orthohantaviruses in humans were done among forestry workers, blood donors and patients with FUO in some countries [Mertens et.al., 2011, Engler et.al., 2013]. However, there is no systematic data on the seroprevalence of orthohantaviruses in humans in Kazakhstan. In our study, we investigated patients with FUO for orthohantaviruses in two non-endemic regions of Kazakhstan, Almaty region and Kyzylorda region. Our results highlighted that many FUO patients had a high level of orthohantavirus reactive IgG antibodies in their serum. IgG antibodies indicate that the host had an orthohantavirus infection in the past. Hence, orthohantaviruses were not the cause of their current FUO situation. Nevertheless, we also identified four IgM positive patients by ELISA. Patients with an IgM positive status are categorised as patients that suffer from an acute infection with the virus. To identify the species causing the human IgG/M reaction immunoblots and IFAs were performed. From the IgM positive sera three samples were reactive with PUUV by immunoblot. Further, we got weak positive reactions for HTNV, SAAV, DOBV, SEOV serotypes by IFA IgM. IgM against orthohantaviruses can exist several months after onset of the disease on a low level that can only be detected by highly sensitive methods such as an IFA [Kruger et.al., 2015, Meisel 2006].

Our results of immunoblotting the IgG positive sera showed bands in 34/178 samples (19.1%) in Almaty and Kyzylorda regions that were indicative to PUUV, HTNV, and DOBV serotypes. On the other hand, IFA results showed in 20/178 samples (11.2%) in Almaty and Kyzylorda regions PUUV and DOBV serotypes.

However, it needs to be kept in mind that a high rate of IgG ELISA positive samples could be related to cross-reactivity, a common dilemma of screening tests. This difference between ELISA and confirmatory assays has been shown in several orthohantavirus seroprevalence studies [Engler et.al., 2013, Sevancan et.al., 2015].

It has to be mentioned that in other published studies on FUO in Kazakhstan there is reported evidence for other pathogens as potential causes of FUO, such as CCHFV, Rickketsia, or TBEV. Also here it has been suggested that numerous infections may remain undiagnosed [Abdiyeva et.al., 2019, Turebekov et.al., 2021].

The clinical manifestation of HFRS exhibits a broad range of symptoms starting from flu-like symptoms to acute renal failure and haemorrhages [Krautkrämer et al., 2013]. In this study the patients

with IgM positive serum samples presented nonspecific clinical signs that can be also attributed to mild forms of the disease [Golovilova et.al., 2007, Jiang et.al., 2016]. This was the first human study to screen for orthohantaviruses by different serology approaches. Therefore, we are able to draw a preliminary picture of the distribution of orthohantaviruses in FUO patients in the selected areas. Some studies showed that the orthohantavirus seroprevalence in humans in Asian countries, for example China, Korea, Thailand, and Singapore prevailed between 0.5% and 33.3%, and in European countries between 0% and 24%. [Bi et al., 2008; Mertens et al., 2011; Jiang, et al., 2016; Xiao et al., 2018; Zou et.al., 2016].

Unfortunately, we could not isolate any virus RNA from these IgM positive serum samples, as the viremia is rather short. Still, we were curious if we are able to identify viral RNA in the serum samples of patients. Hence, we initiated a second human study deliberately seeking for individuals that acutely present symptoms of HFRS in West Kazakhstan region and Almaty city. Indeed, in seven acute HFRS patients expressing high levels of IgM it was possible to isolate RNA from the serum, saliva and urine samples. A subsequent phylogenetic analysis confirmed an infection with PUUV that is closely related to circulating strains in southern Russia and in Sweden. Five positive samples present West Kazakhstan region where HFRS is endemic and we confirmed existence of PUUV by immunoblot and by PCR. However, one positive patient from Almaty city was infected by orthohantavirus. This was in an area more than 2000 km southeast of the endemic area for orthohantaviruses in Kazakhstan. This positive sample originated from the nephrology department where patients are admitted when they show signs of an acute nephrological pathology. There are several reasons for the spread of the virus across the entire country. Beside several natural cases, it might also be accelerated by an increase in transport activities on the roads and newly constructed railways. Driven by the Chinese Belt and Road initiative, Kazakhstan is developing into a globally connected hub and may face the spread of many other pathogens due to human mobility in the near future.

Orthohantavirus infections in humans are always a dead-end for orthohantaviruses in Central Asia. No person-to-person infection with orthohantaviruses has been reported in Eurasia so far. Small mammals are the main vectors of orthohantavirus and they spread it to humans by their excreta. Again, the knowledge of the distribution of orthohantaviruses among small mammals in Kazakhstan is limited. Only few reports demonstrated orthohantaviral antigen in small mammals in Almaty region and one molecular study in Taldykorgan and Karatal demonstrated the existence of TULV in this area [Plyusnina et.al., 2008, Sutyagin et.al., 2014]. However, TULV seems to be not pathogenic to humans. Few reports demonstrated some clinical signs of a TULV infection in immunocompromised patients [Klempa et.al., 2003, Schultze et.al., 2002, Hofmann et.al., 2021] but no severe effects are known to healthy individuals [Mertens et.al., 2011].

Here we screened for orthohantavirus RNA in a variety of rodents in West Kazakhstan, Almaty region and Almaty city. Molecular-biology results of screened individuals were positive for orthohantavirus in 2.4% of the collected small mammals. Sequencing results demonstrated the presence of TULV in these positive samples in different small mammals in West Kazakhstan and also confirm it in the Almaty region. No positive results were identified in Almaty city. We isolated the TULV in two different species. The first is *M.arvalis*, a common host for the virus. The second is *D. nitedula,* a surpisingly uncommon host for TULV. The literature on TULV says that it is found in large numbers in *Microtus* spp. Of the Arvicolinae subfamily and *Lagurus lagurus* [Song et.al.,

2002, Schmidt et.al., 2010]. In our case, *D. nitedula* of the Gliridae family is a novel host for TULV that needs further investigation for a final confirmation. However, by comparing the capture sites of those two infected species it becomes apparent that they had a spatial difference of only 325 meters. The existence of atypical host species as an orthohantavirus reservoir may in fact represent a spill-over infection. This is mostly reported in high incidence areas in Europe, with known circulation across species that reside within the same geographic location [Zou et.al., 2008].

Having a look on the phylogenetic analysis of the sequences of parts of the S segment showed the formation of two distinct clusters of orthohantaviruses, one in West Kazakhstan and the second one in Almaty region. The West Kazakhstan sample has a close evolutionary relationship with the published Samara strain whereas the Almaty region strains (Tekeli and Rudniychiy) shared a close relationship with previously published sequences of *M. arvalis obscurus* sampled in the villages of Karatal and Taldykorgan city, located also in Almaty region [Plyusnina et.al., 2008].

Partial L segment analysis presented similar sequence relationships as the S segment analysis. The TULV L segment sequence from West Kazakhstan region formed its own distinct geographic cluster while the Almaty region sequences formed an individual branch in one big cluster with sequences from China and Turkey. It is highly probable that there exist different geographic lineages of TULV in Kazakhstan transmitted by different subspecies of rodents as recently shown for TULV sequences in Europe. [Schmidt et.al., 2010, Schmidt et.al., 2016]. Orthohantavirus L sequences were described here for the first time in comparison to a previous study that only analysed the S segment [Plyusnina et.al., 2008].

The West Kazakhstan region is an officially endemic region for human cases of HFRS. HFRS can be caused by different orthohantavirus species such as PUUV, SEOV, HNTV or DOBV, with manifestations at different degrees of severity. As an example, TULV is usually described to be a very benign virus only causing symptoms in immunocompromised patients. Further, it should be mentioned that in some cases TULV associated human infectious can cross-react with PUUV [Meisel et.al., 2006]. A focus-reduction neutralisation test (FRNT) would be a specific confirmatory and gold standard serological test and can be used to discriminate between different species of orthohantaviruses, but unfortunately, in our study we could not type the serum samples by FRNT as this method is not available in Kazakhstan.

PUUV associated HFRS is suspected to be prevalent in West Kazakhstan region as West Kazakhstan region borders with Orenburg and Samara regions of Russian Federation where PUUV is endemic [Kariwa et.al., 2009, Jonsson et.al., 2010]. But this assumption was never corroborated by molecular-biological investigations in host reservoirs as well as human samples.

Interestingly, our study on reservoir hosts only showed the existence of TULV only in one specimen. Albeit the clinical manifestations of hospitalized patients with HFRS in the West Kazakhstan region is described as mostly moderate (signs of haemorrhages and acute renal failures), it is still highly improbable that all of these cases are caused by PUUV [Zakharov et.al., 2010, Grazhdanov et.al., 2014]. Hence, we have to assume that there is a reservoir of other orthohantavirus species that still remains unidentified so far. Potential reasons that only TULV but no PUUV was detected might rest in the choice of the sampling sites as in West Kazakhstan the territory is vast and we only investigated some part of the endemic districts in a rather limited hunt for the typical PUUV

carrier *Myodes glareolus*. Furthermore, climate and environmental conditions are important factors that can greatly modify the spread of this virus family.

2.7 Conclusion

Here, we conducted for the first time in the Republic of Kazakhstan a serology study of orthohantaviruses in patients with FUO and in patients with suspected cases of HFRS using different serology tools. In parallel small mammals were captured and investigated for infection rates of orthohantavirus using contemporary molecular-biology methods.

Our study identified the existence of antibodies against several serotypes of orthohantaviruses such as PUUV, HNTV, DOBV in patient's serum samples with FUO which corresponds to an exposure to orthohantaviruses in these patients in the past. Excitingly, these patients were identified in two previously non-endemic regions (Almaty and Kyzylorda) of Kazakhstan. Moreover, our results showed the presence of IgM antibodies against orthohantavirus in patients with FUO in Almaty region. Moreover, serology and molecular-biology study of patients with suspected cases of HFRS could confirm presence of PUUV in West Kazakhstan region and in one specimen of Almaty city. These results highlight that the awareness about orthohantaviruses among treating doctors in even mild forms of FUO is important. Dissemination of suitable case definitions will be important so that health care workers will be able to correctly identify potential cases, and as a consequence access to reliable diagnostics needs to be assured.

Our results further support that monitoring of host reservoirs by serology and molecular-biology methods are a valuable approach to predict human infections and to design preventive measures against HFRS. The frequent presence of TULV in rodents in Almaty region and West Kazakhstan region gives a hint that orthohantaviruses are more spread in the Central Asian country of Kazakhtsan than previously assumed.

The initial rudimentary studies presented here in this thesis highlight the importance to initiate more lateral and longitudinal studies of orthohantaviruses in patients, and, equally important, in their natural hosts. Furthermore, the availability of contemporary laboratory tools to diagnose emerging diseases in humans as well as in host reservoirs are indispensable for public health and a prime example of a One Health approach.

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4. Publications

4.1 Paper A: Serological investigation of orthohantaviruses in patients with fever of unknown origin in Kazakhstan

Tukhanova, N., Shin, A., Abdiyeva, K., Turebekov, N., Yeraliyeva, L., Yegemberdiyeva, R., Shapiyeva, Zh., Froeschl, G., Hoelscher, M., Wagner, E., Rösel, K., Zhalmagambetova, A., Musralina, L., Frey S., Essbauer, S. (2020). Serological investigation of orthohantaviruses in patients with fever of unknown origin in Kazakhstan. Zoonoses and Public Health, 67: 271–279.

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ORIGINAL ARTICLE

WILEY

Serological investigation of orthohantaviruses in patients with fever of unknown origin in Kazakhstan

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Abstract

Objective: Orthohantaviruses are geographically widely distributed and present various clinical manifestations from mild symptoms to the severe form of haemorrhagic fever with renal syndrome (HFRS) in Eurasia. Official registration of HFRS in Kazakhstan started in the year 2000. However, the true prevalence of human infections by orthohantaviruses within Kazakhstan is unknown. The aim of this study was to investigate of the seroprevalence of orthohantavirus infections in patients with fever of unknown origin (FUO) in two regions, Almaty and Kyzylorda region.

Methods: Paired serum samples from 802 patients with FUO were screened for the presence of orthohantavirus IgG and IgM antibodies by ELISA. Positive samples were further tested by immunoblotting and indirect immunofluorescence tests (IIFT) to determine the respective orthohantavirus serotypes. Suspected acute serum samples were additionally checked by a RT-PCR to identify viral RNA.

Results: In total 178/802 (22.2%) serum samples reacted with orthohantavirus IgG antibodies and 4/802 (0.5%) with IgM antibodies. All positive samples were tested by immunoblotting which resulted in 2.9% positive samples with IgG antibodies against Puumala (PUUV), Hantaan (HTNV) and Dobrava (DOBV) virus serotypes in Almaty region and 5.4% to PUUV and DOBV serotypes in Kyzylorda region, respectively. In the IFFT, 1.9% positive samples from Almaty and 3.1% from Kyzylorda were confirmed for PUUV and DOBV serotypes. Out of four IgM ELISA positive samples only three were positive against PUUV in the immunoblot and showed weak positive reactivity for the Saaremaa (SAAV), PUUV and HTNV serotypes in the IFFT.

Conclusions: This study demonstrates the presence of orthohantavirus infections among patients with FUO in Kazakh regions that were so far considered as non-endemic. The healthcare system needs to be prepared accordingly in order to be capable of detecting cases and providing adequate management of patients.

KEYWORDS
fever of unknown origin, Kazakhstan, orthohantavirus, serology

1 | INTRODUCTION

Orthohantaviruses (family *Hantaviridae*, order *Bunyavirales*) are RNA viruses, dispose of a lipid envelope and form spherical or oval viri- ons of 80 to 120 nm. The virus genome consists of three segments of a single-stranded negative orientated RNA (Vaheri, Henttonen & Voutilainen, 2013; Vaheri et al., 2013). Presently, according to the actual report of the International Committee on Taxonomy of Viruses there are at least 41 species of orthohantaviruses (ICTV, 2018).

Orthohantaviruses are detected in many species of small mammals throughout the world. The viruses are mainly circulating in rodents such as *Arvicolinae* and *Murinae*, but are sometimes also found in bats or shrews (Essbauer & Krautkrämer, 2015; Krautkrämer, Zeier, & Plyusnin, 2013). Humans become infected by contact with rodents or their products: urine, saliva and faeces and by inhalation of aerosols containing virus (Hart & Bennett, 1999; Johnson, 2001; Lednicky, 2003). In general, orthohantaviruses can induce two distinct types of diseases: hantavirus cardiopulmonary syndrome (HCPS) in the Americas and haemorrhagic fever with renal syn- drome (HFRS) in Europe and Asia (Essbauer & Krautkrämer, 2015; Schmaljohn & Hjelle, 1997).

Haemorrhagic fever with renal syndrome is caused by the strains Hantaan orthohantavirus (HTNV), Seoul orthohantavirus (SEOV), Puumala orthohantavirus (PUUV), Dobrava-Belgrade orthohantavirus (DOBV), Tula orthohantavirus (TULV) and Amur orthohantavirus (AMRV), (Papa et al., 2016; Schmaljohn & Hjelle, 1997; Vapalahti et al., 2003; Vaheri et al., 2013). Clinical and epidemiological features of infection may be different for various orthohantavirus strains. TULV infection is mostly mani- fested in mild clinical forms, only two cases were reported from Germany, also with no fatality (Klempa et al., 2003; Schultze, Lundkvist, Blauenstein, & Heyman, 2002). PUUV and SEOV both cause mild clinical manifestations including renal symptoms (PUUV: *nephropathia epidemica*) and mortality is low, between 1% and 2%. Four genotypes have been identified in DOBV: For the Dobrava genotype, clinical manifestations range from mild to severe with a case fatality rate of 10%–12%. For the Kurkino genotype, clinical manifestations are mild to moderate and the case fatality rate is 0.3%–0.9%. For the Saaremaa (SAAV) geno- type asymptomatic infections are known, and data on lethality are not available. Infections with the Sochi virus genotype are moderate to severe with a case fatality rate of more than 6%. Finally, HTNV induces the most severe clinical manifestations in the spectrum of HFRS and goes on with a higher lethality rate of 10%–15% (Krautkrämer et al., 2013; Essbauer & Krautkrämer, 2015).

The vastness of the territory of Kazakhstan harbours many natural foci of zoonotic diseases. Only few zoonotic diseases have been studied and for some there are only indications based on clinical symptoms. Besides, haemorrhagic fever can be caused by orthohantaviruses, which is of interest for the health sur- veillance system in Kazakhstan. The first human cases of HFRS were detected and laboratory—confirmed (IIFT, ELISA IgG paired serum samples) in the village of Zharsuat in the Burlinskiy dis- trict in the West Kazakhstan region in the year 2000. From 2000 to 2018, 245 cases of HFRS were clinically registered and se- rologically confirmed by IIFT/ELISA in the West Kazakhstan re- gion (Bekmukhambetov, 2012; Zakharov, Grazhdanov, Zakharov, & Nazhimova, 2010). Investigations of rodents as reservoir host are limited in Kazakhstan. Only one report describes TULV found in tissue samples of *Microtus arvalis* in the Almaty region (Taldykorgan and Bakanas), but TULV is usually not pathogenic in humans or causes only mild diseases (Plyusnina, Laakkonen, Niemimaa, Henttonen, & Plyusnin, 2008). From 2001 to 2011, 49,676 small mammals were screened for the orthohantavirus antigen by ELISA in West Kazakhstan region. In four rodent species, 758 positive results were obtained (Grazhdanov et al., 2014). Nowadays the West Kazakhstan region is designated as an endemic area for orthohantaviruses. Unfortunately, no in- formation concerning other regions of Kazakhstan is available. Orthohantavirus infections are expected to be underdiagnosed as these do not uncommonly lead to atypical or mild illness and diagnostic testing is difficult (Bi, Formently, & Roth, 2008; Sevencan et al., 2015). The exact prevalence of orthohantavirus infections in cases of FUO within Kazakhstan is unknown. The aim of the study was to investigate the seroprevalence and se- rotype of orthohantavirus infections in patients with FUO in two regions of Kazakhstan.

IMPACTS

- FUO can be caused by a broad variability of zoonotic infectious agents such us orthohantaviruses. There exist no data on orthohantaviruses in patients with FUO in Kazakhstan.
- We demonstrate a high seroprevalence against orthohantaviruses in two regions of Kazakhstan. Additionally, we showed acute infections and that the present virus type might be Puumala orthohantavirus.
- Physicians in Kazakhstan should be aware that clinical symptoms starting with mild fever could be caused by orthohantaviruses. As rodents are a reservoir for orthohantaviruses further studies on these reservoir animals should be initiated.

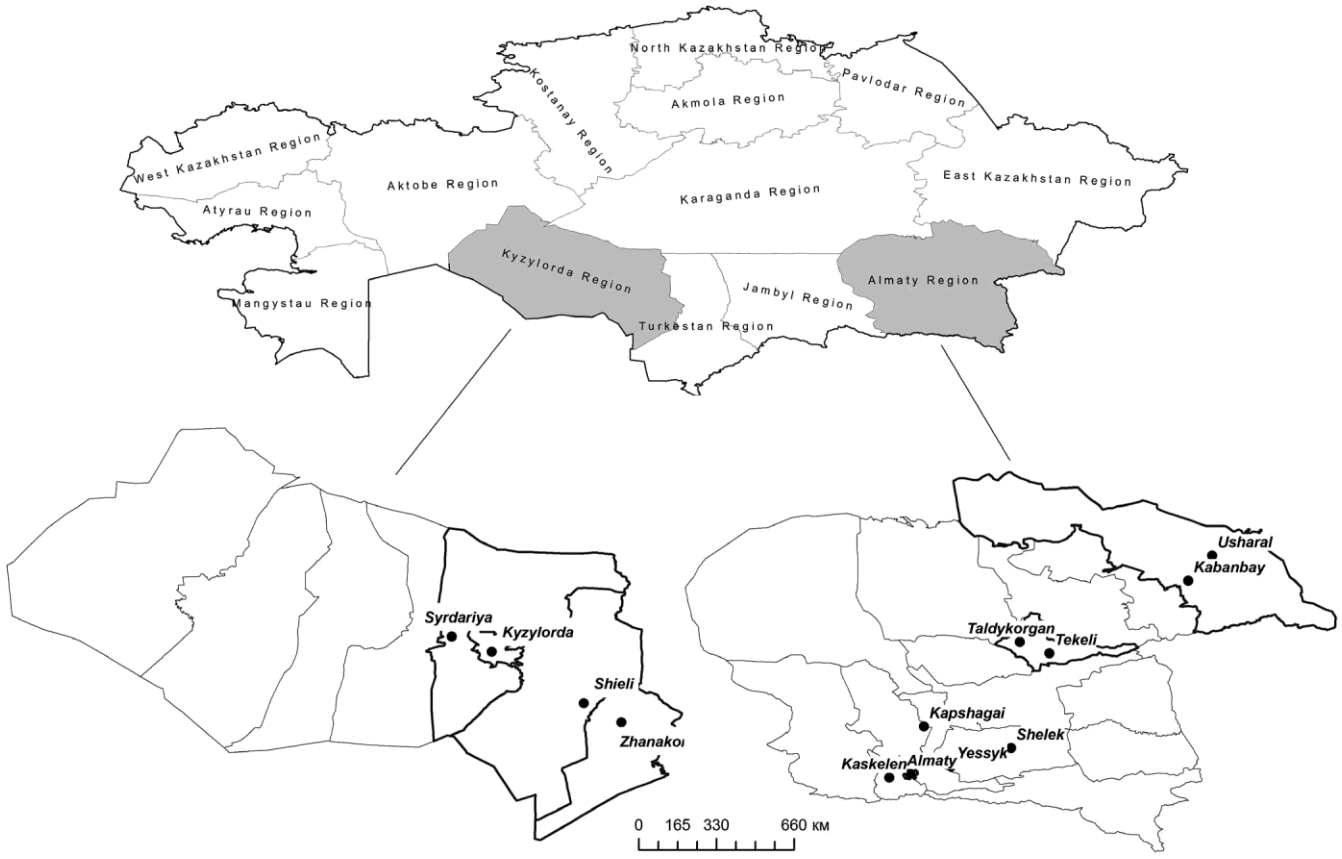

FIGURE 1 Geographical location of sampling points in two regions of Kazakhstan: Almaty and Kyzylorda

2 | MATERIALS AND METHODS

2.1 | Study design

A cross-sectional descriptive study was set up in 2015–2016 among patients with FUO in Kazakhstan in the Almaty and the Kyzylorda region (Figure 1). In these two regions, 13 hospitals were selected to conduct various studies in patients with FUO with a focus on rodent- and arthropod-borne infections (Abdiyeva et al., 2019).

2.2 | Ethics approval

This study was performed in accordance with the Kazakhstan local ethics committee at the Kazakh National Medical University in Almaty, Kazakhstan (opinion numbers 194–15, 564–18) and Ludwig-Maximilians-Universität in Munich, Germany (opinion numbers 16–175, 18–631). Blood sampling was conducted after signed informed consent. From participants under 18 years of age, the signed informed consent was taken from both parents or guardians and the underage participant.

2.3 | Sample collection

Responsible doctors identified hospitalized patients with FUO at the 13 hospitals included in the Almaty and Kyzylorda region. FUO was defined as presenting with sub-febrile or febrile temperatures. Fever was defined by taking the temperature via tympanic measurement and lasting at least for three days. Rhinitis or any other laboratory-confirmed diseases represented exclusion criteria. Participants of both sexes and of age ≥15 years were included in the study. All participants signed an informed consent form. A standardized questionnaire was completed using a face-to-face interview method. The questionnaire included 47 questions with sociodemographic, living and housing, livestock, vector habitat and clinical symptoms modules.

Blood sampling was performed twice: the first serum sample was taken on the first day of hospitalization; the second serum sample was taken 10–14 days later. Paired blood samples were centrifuged, and sera were split into aliquots and conserved at −20°C for further serological testing. The required amount of serum was heat-inactivated (56°C, 60 min) before further being processed in the serological study.

2.4 | ELISA-screening

All serum samples were tested for the presence of orthohantavirus IgG and IgM by a commercial ELISA (Novatec Immunodiagnostica). The ELISA plates were read by optical density (OD) with an ELISA plate reader (Infinite F50, Tecan). OD values were measured at 450 nm with 620 nm as a reference (Novatec, Immundiagnositca GmbH, NovaLisa HANG0670 Manual). Results were calculated in Novatec Units (NTU) as the patients mean absorbance value multiplied with ten and divided through the mean

cut-off. Patients with a NTU < 8 were negative, patients with NTU > 11 were designated as serum samples from patients that had contacts with the antigen and therefore as positive. Serum samples with a NTU between 9 and 11 were judged as equivocal and repeated. If the result was equivocal again the sample was judged as negative.

All second serum samples were screened for IgG antibodies. To find out if it was an acute or a previous infection, all IgG positive second serum and the corresponding first serum samples were further tested for IgG antibodies and gained NTU were compared. If the first serum was negative for IgG antibodies, the first serum was tested against IgM antibodies. In the case that the first serum was negative for IgM antibodies, this first serum was further tested by molecular methods as well as all IgM-positive first serum samples. If both paired serum was positive (NTU > 11) for IgG antibodies and if the difference was ≤2, it was declared as being negative for an acute infection. In the case the difference was >2 a titration with serial dilution was performed (1:101, 1:201, 1:401, 1:801). A 4-fold and higher titre difference between second and first serum was estimated as an acute infection.

2.5 | Serotyping

To verify the orthohantavirus serotypes of positive serum samples, IgG and IgM were further investigated by IgG and IgM immunoblotting tests (Microgen recomLine HantaPlus) and IgG and IgM IIFT (Euroimmun) according to the manufacturers' protocols.

The immunoblotting test provides a strip assay for the detection of human antibodies of the IgG and IgM classes for five different orthohantavirus serotypes and one phlebovirus: PUUV, HTNV, DOBV, Seoul virus (SEOV) and Sandfly virus. The test strips were visually evaluated from (−) to (+++). Low intensity (+) to strongly (++/+++) coloured bands were interpreted to indicate positively.

Anti-orthohantavirus IIFT for the determination of antibodies class IgG and IgM were performed by using commercial slides of the Hantavirus Mosaic 2 Eurasia (Euroimmun) for HTNV, PUUV, SEOV, SAAV, DOBV serotypes with 1:10 and 1:100 dilutions. Results were evaluated independently by two persons using a fluorescence microscope (MicroOptix MX 300).

2.6 | Molecular investigations

RNA was extracted from 140 µl serum sample using the commercial kit QiAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Presence of RNA was examined by a panHanta reverse transcriptase qPCR (Mossbrugger, Felder, Gramsamer, & Wölfel, 2013) in a Qiagen One-Step RT-PCR mix on a Rotor-Gene Q cycler (Qiagen).

2.7 | Data analysis

The statistical analysis of the results was performed using STATA (R) 15.1 (StataCorp, 2017). Chi-square test was calculated for the estimation of the association between risk factors and seropositivity. *p*-values of ≤ .05 were considered as statistically significant. Univariate analysis was conducted to calculate odds ratio (OR) and 95% confidence interval (CI) to identify possible risk factors.

3 | RESULTS

During the study period 2015 and 2016, 950 patients with FUO presented in the 13 hospitals of the two regions in Kazakhstan. In summary, 148 patients had to be excluded per protocol for not providing paired serum samples or completing the study questionnaires. Out of the remaining 802 paired serum samples, orthohantavirus specific IgG antibodies were found by ELISA in 22.2% (178/802) of the study subjects. In four serum samples, 0.5% (4/802) positive orthohantavirus IgM antibodies were detected indicating the suspicion of an acute infection (Table 1).

All 178 IgG-positive serum samples were further checked for titration. In 130 from 178 serum pairs (73.0%) OD was ≤2 units and therefore these were not titrated. Out of 178 serum pairs, 31 (17.4%) showed low titres (1:101) and 17 serum pairs (9.5%) showed medium titres (1:201, 1:401) by titration and were evaluated as having had already previous exposure. There were no samples with high titres (Table 2).

All orthohantavirus ELISA IgG-reactive (n = 178) and IgM-reactive (n = 4) samples were further tested by immunoblotting assay (IgG and IgM) and IIFT to identify circulating serotypes of orthohantaviruses. Among 178 ELISA IgG-positive serum samples the reactivity for PUUV, HTNV, DOBV was confirmed by IgG immunoblotting test in 20 serum samples (11.2%) and by IgG IIFT for PUUV, DOBV serotypes in 34 serum samples (19.1%, 5 positive in 1:10, 15 positive in 1:100 dilution). Three of four tested serum samples were positive for PUUV serotype by IgM immunoblotting

TABLE 1 Results of the orthohantavirus serology study by ELISA IgG and IgM among patients with FUO in the Almaty and Kyzylorda region 2015–2016

Regions	Total number of tested serum samples	Number of IgG positive orthohantavirus samples 2nd/1st serum (%)	Number of IgM positive orthohantavirus samples 1st serum (%)	Number of negative serum samples (%)
Almaty	378	80 (21.2)	4 (1.0)	294 (77.8)
Kyzylorda	424	98 (23.1)	0	326 (76.9)
Total	802	178 (22.2)	4 (0.5)	620 (77.3)

TABLE 2 Results of tested anti-orthohantavirus IgG positive paired serum samples on ELISA

ELISA IgG result (2nd/1st serum)	Number of serum samples (%)
Low titre (1:101/1:101)[a]	130 (73.0%)
Low titre (1:101/1:101)	31 (17.4%)
Moderate titre (1:201-1:401/1:201-1:401)	17 (9.5%)
High titre (1:801/1:801)	0
Total	178

[a]If the optical density between second and first serum was ≤OD units, these were not titrated.

testing. In one case no serotype identification could be seen. IIFT showed in three serum samples a weak positive reactivity in 1:10 and 1:100 dilution with SAAV, PUUV, DOBV, SEOV and HTNV serotypes (Table 3).

The four serum samples indicating an acute orthohantavirus infection originated from the Almaty region from three hospitals (Yessyk hospital: 2 positive patients (YEN1-200 50, YEN1-200 59), Almaty hospital: one positive patient (ALM-800 108), Tekeli hospital: 1 positive patient (ESK-600 004)). Of the four positive participants, three were female with ages of ages 22, 33 and 51 and one male at the age of 19. Of the IgM-positive participants, two individuals lived in rural and two in urban areas (p = 1.000).

TABLE 3 Results of orthohantavirus immunoblotting and IIFT IgG and IgM among patients with FUO in the Almaty and the Kyzylorda region 2015–2016

		IgG (n = 178)		IgM (n = 4)	
Regions	**Serotype**	**Immunoblot test**	**IIFT**	**Immunoblot test**	**IIFT**
Almaty	PUUV	7	6	3	3[a]
	HTNV	3	0	0	0
	DOBV	1	1[a]	0	0
	(%)	2.9	1.9	1.0	1.0
Kyzylorda	PUUV	15	9	0	0
	DOBV	8	4	0	0
	(%)	5.4	3.1	0	0
Total (%)		34 (19.1)	20 (11.2)	3 (1.0)	3 (1.0)

Abbreviations: DOBV, Dobrava orthohantavirus; HNTV, Hantaan orthohantavirus; PUUV, Puumala orthohantavirus.

[a]Reactivity with Saaremaa, Puumala, Dobrava, Seoul and Hantaan serotypes.

TABLE 4 Results of ELISA, immunoblotting test, IIFT and RT-PCR positive orthohantavirus IgM serum samples[a]

Serum samples		YEN1 200–050	YEN1 200–059	ALM 800–108	ESK 600–004
ELISA IgM	1st serum	+	+	+	+
Immunoblotting IgM	PUUV	+	+	+	–
	SINV	±	±	–	–
	HNTV	±	±	–	–
	DOBV	±	±	±	–
	SEOV	–	–	–	–
	SFV	–	–	–	–
IIFT IgM (1:10, 1:100)	HNTV	±	–	–	–
	PUUV	±	±	±	–
	SEOV	±	–	–	–
	SAAR	–	±	±	–
	DOBV	–	–	±	–
	Non infected cells	–	–	–	–
Reverse transcriptase qPCR	1st serum	Negative	Negative	Negative	Negative

Abbreviations: DOBV, Dobrava orthohantavirus; HNTV, Hantaan orthohantavirus; PUUV, Puumala orthohantavirus; SAAR, Saaremaa orthohantavirus; SEOV, Seoul orthohantavirus; SFV, Sandfly virus; SINV, Sin Nombre orthohantavirus.

[a]+ positive (low intensity), +/− weak positive (very low intensity).

Concerning the daily activities investigated half of the participants did garden and fieldwork (p = .864), and three of them had seen rodents (p = .213). The clinical manifestations of positive IgM subjects showed fever (n = 4), headache (n = 3), weakness (n = 2), arthralgia (n = 2), back pain (n = 1) and nose congestion (n = 1). In total three of the four IgM positive ELISA serum samples were confirmed by Immunoblotting tests for the PUUV serotype (YEN1-200 50, YEN1-200 59, ALM-800 108) with low intensity (+) coloured bands. All these three samples showed weak positive result in the IIFT with 1:10 and 1:100 dilution to SAAV, PUUV, DOBV, SEOV and HTNV serotypes. All IgM-positive serum sam- ples were additionally tested by RT-PCR to detect RNA of ortho- hantaviruses. In none of these samples orthohantavirus RNA was detected (Table 4).

To assess the potential risk factors for orthohantavirus infections, a univariate logistic regression was performed on in the ELISA IgG-positive serum samples. No significant association could be identified between risk factors such as sex, last nature trip, house location in urban or rural area or the fact that the person had seen rodents with seropositivity. Working in a garden and in the field, as often 1.7 and as always 2.9, increased risk of seropositivity but it was not significant (p = .05). By the way, patients with age ≤50 had 2.26 times more seropositivity compared with the age >50 and it was statistically significant. On the other hand, there were no risk factors identified on positive immunoblot IgG serum and IIFT IgG serum samples.

4 | DISCUSSION

Orthohantavirus infections are globally wide-spread and during the last two decades are receiving more attention as a relevant public health problem. In Kazakhstan, the investigation of orthohantaviruses has been focusing so far on the West Kazakhstan region as there were previous human cases recognized by clini- cal patterns which were also laboratory confirmed. Nevertheless, some rodent investigations revealed that the natural foci of orthohantaviruses are located between the West Kazakhstan region and Orenburg, the Samara regions of the Russian Federation (Alexeyev, Elgh, Zhestkov, Wadell, & Juto, 1996; Aminev, Korneev, Slobodenyuk, & Solovich, 2014; Grazhdanov et al., 2013). Annual registrations of HFRS in the West Kazakhstan region began in 2000, and a high incidence rate of 16 per 100,000 inhabitants was described in 2005 (Grazhdanov et al., 2014). In the West Kazakhstan region from 2001 onwards, the investigation of reservoirs started. These showed the orthohantavirus antigen by ELISA in different species of rodents: bank voles, common voles, forest mice and house mice (Grazhdanov et al., 2014). Another report demonstrated that rodent tissue suspensions collected in the Almaty region Dzungarian, in the Alatau mountains in 2010–2016, 2.2% (15/684) were positive for orthohantavirus antigens using ELISA (Test system: Hantagnost, Russia), (Sutyagin, Belyaev, Kim, & Berdibekov, 2017).

However, there exist no systematic data on the seroprevalence of orthohantaviruses in humans in Kazakhstan. Some studies showed that the orthohantavirus seroprevalence in Asian countries, for ex- ample China, Korea, Thailand and Singapore prevailed between 0.5% and 33.3%, and in European countries between 0% and 24%. (Bi et al., 2008; Mertens et al., 2011; Jiang, Zhang, et al., 2016; Xiao et al., 2018; Zou, Chen, & Sun, 2016).

In Kazakhstan, various zoonotic agents have been suspected to be endemic that can cause FUO with mild clinical presentations. Investigations of patients with FUO can provide adequate information for the public health priority setting. However, in resource-limited settings such as in Kazakhstan, the needed high-quality laboratory diagnostics are not or only insufficiently established. Parallel investigations of the same FUO samples used in this study for other arthropod-borne infectious showed that some serum sam- ples with confirmed orthohantavirus IgG antibodies were reactive also for other agents: for Crimean-Congo haemorrhagic fever virus (CCHFV), six IgG serum samples, for *Rickettsia spotted fever* group ELISA (IgG), 13 serum samples and for *Rickettsia typhus* group ELISA, 15 serum samples. However, none of the patients that were orthohantavirus IgM positive had simultaneously antibodies against CCHFV, *Rickettsia of spotted fever* group and *Rickettsia of typhus* group (Abdiyeva et al., 2019).

This study presents the first seroprevalence study of orthohantavirus infection among patients with FUO in two regions of Kazakhstan using a combination of serological assays. Our study identified an acute orthohantavirus infection in four serum samples on ELISA and three of them reacted with PUUV serotype by immunoblotting and showed a weak positive reaction for PUUV, HTNV, SAAV, DOBV, SEOV serotypes by IIFT. However, IgM titres against orthohantaviruses can stay positive for several months after the onset of disease, which relativizes our assumptions on acute cases in our patient group (Krüger, Figueiredo, Song, & Klempa, 2015; Meisel et al., 2006). In this study, we could not type the pa- tient's serum by FRNT as such tests are currently not available in Kazakhstan. RT-PCR has been done for the four suspected acute serum samples. However, viremia phases during orthohantavirus infections in humans are short and present before IgM antibodies are present, which could also be the case in this study (Krautkrämer et al., 2013; Krüger et al., 2015). Clinical manifestations of HFRS are characterized by acute renal failure followed by haemor- rhage and flu-like symptoms such as fever, headache, abdominal/ back pain and range from subclinical or mild to severe symptoms (Krautkrämer et al., 2013). In the present study, patients with IgM- positive serum samples developed unspecific clinical signs that can also be attributed to a mild form of the disease (Golovljova et al., 2007; Jiang, Du, Wang, Wang, & Bai, 2016). Moreover, orthohanta- virus IgM levels were investigated instead to determine suspected acute cases among patients with FUO. Generally, this study showed that IgM-positive patients were more females than males, but this was not statistically significant as given by the small case numbers (Latronico et al., 2018; Sevencan et al., 2015). We did not find a relationship with some risk factors such as living place, garden or

fieldwork or the observation of rodents with IgM-positive cases (Botros et al., 2004).

The most practical approach of orthohantavirus infections are based on ELISA IgG antibodies as it was also used for seroepidemiological studies. Likewise, in some seroepidemiological studies ini- tial screening was also done by ELISA followed by further analyses using Immunoblot, IIFT and FRNT assays (Hukic et al., 2010; Zou et al., 2016). In this study, screened serum samples showed 22.2% positive results for IgG antibodies to orthohantaviruses by ELISA. Immunoblotting and IIFT confirmed all samples considered as positive. According to the results of the immunoblotting, the orthohantavirus IgG exposure among people with FUO was estimated to be 2.9% in the Almaty region and 5.4% in the Kyzylorda region with different serotypes (PUUV, HTNV and DOBV), by IIFT 1.9% in Almaty and 3.1% in Kyzylorda regions (PUUV and DOBV), respectively. In our study, the high rate of positive IgG antibodies by ELISA shown here could be false-positive, originating from the sensitivity of the screening test. Moreover, the difference between ELISA and confirmatory assays has been shown in several orthohantavirus seroprevalence studies (Engler et al., 2013; Sevancan et al., 2015). The different results by immunoblotting and IIFT can be explained by sensitivity (immunoblotting—96.1%, IIFT—99%) and specificity (immunoblotting—100%, IIFT—98%) of the used assays (mikrogen.de, euroimmun.de). However, immunoblotting assay is used as more suitable diagnostic and confirmatory test (Engler et al., 2013; Escadafal et al., 2012). In the Almaty region some rodent studies were conducted, in which in some areas rodents were found to be positive for orthohantaviruses, but no clinical case of HFRS has officially been registered in this region so far (Plyusnina et al., 2008; Sutyagin et al., 2017). Notable is that at some parts of the border between the Almaty region and China the orthohantavirus seroprevalence has been re- ported to range between 1% and 12% (Avšič Županc & Korva, 2014; Bi et al., 2008). So far in the Kyzylorda region, orthohantaviruses have not been studied in human cases. We are therefore the first to promote that orthohantaviruses seem to circulate in this region.

In agreement with previous studies in the present study, no significant association was identified between risk factors concerning sex, last nature trip, house location in urban or rural area or the fact that the patient had seen rodents with the IgG ELISA seropositivity (Botros et al., 2004; Christova et al., 2017; Sin et al., 2007). In our study, garden fieldwork and the age ≤50 years was the risk factor associated with IgG seropositivity on ELISA. Similar data of outdoor activities were demonstrated in a study from Sweden (Gherasim et al., 2015). It is probable that such findings are due to having had contact with rodents or their excreta during gardening.

In conclusion, these data present the first seroprevalence study of orthohantavirus infections in humans with FUO in Kazakhstan. The data obtained show that the diagnostics of orthohantaviruses among individuals with FUO is important given the potential se- vere course of the presentation and the specific treatment options. However, in many cases, the initial presentation with mild forms of the disease with fever and flu-like symptoms may render the differ- ential diagnosis a challenge. So far also data on orthohantaviruses in rodents in Kazakhstan are limited. Additional studies in rodents and humans are necessary in order to be able to better characterize the circulation of virus strains in the region.

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CONFLICT OF INTEREST

None to declare.

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4.2 Paper B: Molecular characterisation and phylogeny of Tula virus in Kazakhstan

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MDPI

Article

Molecular Characterisation and Phylogeny of Tula Virus in Kazakhstan

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Abstract: Orthohantaviruses are zoonotic pathogens that play a significant role in public health. These viruses can cause haemorrhagic fever with renal syndrome in Eurasia. In the Republic of Kazakhstan, the first human cases were registered in the year 2000 in the West Kazakhstan region. Small mammals can be reservoirs of orthohantaviruses. Previous studies showed orthohantavirus antigens in wild-living small mammals in four districts of West Kazakhstan. Clinical studies suggested that there might be further regions with human orthohantavirus infections in Kazakhstan, but genetic data of orthohantaviruses in natural foci are limited. The aim of this study was to investigate small mammals for the presence of orthohantaviruses by molecular biological methods and to provide a phylogenetic characterization of the circulating strains in Kazakhstan. Small mammals were trapped at 19 sites in West Kazakhstan, four in Almaty region and at seven sites around Almaty city during all seasons of 2018 and 2019. Lung tissues of small mammals were homogenized and RNA was extracted. Orthohantavirus RT-PCR assays were applied for detection of partial S and L segment sequences. Results were compared to published fragments. In total, 621 small mammals from 11 species were analysed. Among the collected small mammals, 2.4% tested positive for orthohantavirus RNA, one sample from West Kazakhstan and 14 samples from Almaty region. None of the rodents caught in Almaty city were infected. Sequencing parts of the small (S) and large (L) segments specified Tula virus (TULV) in these two regions. Our data show that geographical distribution of TULV is more extended as previously thought. The detected sequences were found to be split in two distinct genetic clusters of TULV in West Kazakhstan and Almaty region. TULV was detected in the common vole (*Microtus arvalis*) and for the first time in two individuals of the forest dormouse (*Dryomys nitedula*), interpreted as a spill-over infection in Kazakhstan.

Keywords: orthohantavirus; rodents; Republic of Kazakhstan; Tula virus

1. Introduction

The genus *Orthohantavirus* (family *Hantaviridae*, order *Bunyavirales*) includes zoonotic pathogens. This group of viruses plays an important role in causing human diseases worldwide. Orthohantaviruses are single-stranded negative polarity RNA viruses, and the genome consists of three segments. The large (L) segment encodes a viral RNA-dependent RNA polymerase, the medium (M) segment encodes the glycoprotein precursor (GPC), which is processed to the glycoproteins Gn and Gc, and the small (S) segment encodes the nucleocapsid (N) protein [1].

Small mammal species are a reservoir for orthohantaviruses. Orthohantaviruses are presently known to infect rodents (subfamilies Murinae, Arvicolinae, Sigmodontinae, and Neotominae), but are also detected in different shrews and moles [2–4]. In Eurasia, humans are infected either by rare direct contact or indirectly by inhalation of orthohantaviruses containing dust from dried excreta [5,6].

Old World orthohantaviruses can cause haemorrhagic fever with renal syndrome (HFRS) and are mainly transmitted by members of the Murinae and Arvicolinae subfamilies [2,3]. In Europe, the main causative agent of HFRS is Puumala virus (PUUV) causing nephropathia epidemica (NE), a mild form of HFRS. A mild to severe form of HFRS is caused by Dobrava-Belgrade virus (DOBV). In Asia, the most relevant species is Hantaan virus (HNTV) that causes a severe form of HFRS. Seoul virus (SEOV) is distributed worldwide and can cause a moderate form of HFRS [6–9]. Pathogenicity of Tula virus (TULV) to humans is limited, only few reports of human cases were described in Europe [10–13], despite the fact that TULV is found in Asia and Europe. In North America, the TULV-related Prospect Hill virus was identified in a *Microtus* species (*M. pennsylvanicus*) but no human infections have been reported here either [2,7,14,15].

The Central Asian Republic of Kazakhstan has a vast territory and contains several types of landscapes such as forest-steppes, steppes, semi-deserts, deserts, and mountain ranges [16,17]. In these different geographic settings, Kazakhstan has numerous natural foci of important zoonotic pathogens such as *Yersinia pestis, Bacillus anthracis, Francisella tularensis, Leptospira, Listeria monocytogenes*, tick-borne encephalitis virus (TBEV), Crimean-Congo haemorrhagic fever virus (CCHFV), and orthohantaviruses [17,18].

An investigation of small mammals on the Dzungarian Alatau mountain range in Almaty region in 1990–1993 showed that some rodents contain orthohantavirus antigens ($n = 644$, 5.3%) [19]. Twenty years later, a study conducted in the same region using antigen assays found traces of orthohantavirus antigens in 2.2% of investigated tissue suspensions of rodents collected in 2010–2016 [20,21]. Furthermore, the existence of Tula virus was proven in tissue samples of *Microtus arvalis* in Almaty region (periphery of Taldykorgan city and Karatal village) [22].

The first human case of HFRS was detected in the year 2000 in the Zharsuat village in the Borili district, a part of the West Kazakhstan region [23,24]. Further investigations of host reservoirs were started, and from 2001 to 2011 almost 50,000 small mammals including 30 species were screened for the presence of orthohantavirus antigen. A total of 1.53% of different species, mostly *Myodes glareolus*, *Microtus arvalis, Microtus minutus*, *Apodemus uralensis*, and *Mus musculus* were positive. Therefore, so far, natural foci of orthohantaviruses were described in the four northern districts of the West Kazakhstan region (Borili, Bayterek, Shyngyrlau, and Terekti) and very preliminary in the Aktobe region [25,26]. However, in all investigations on orthohantaviruses in West Kazakhstan, contemporary molecular methods were never applied.

To date, there have been no officially registered human cases of HFRS in the Almaty region. However, an investigation of patients with fever of unknown origin (FUO) in Almaty and Kyzylorda regions showed orthohantavirus-reactive antibodies in sera of

patients. This indicates that orthohantaviruses might also be endemic in the southeast of Kazakhstan [23].

The aim of this study was to investigate small mammals for the presence of orthohantaviruses by molecular biological methods in the Almaty region, including Almaty city and in West Kazakhstan, representing an officially endemic region for orthohantavirus infections in humans.

2. Materials and Methods

2.1. Study Setting and Rodent Sampling

Small mammals were trapped in 2018 and 2019 in West Kazakhstan (Bayterek, Borili, Terekti, and Taskaly districts: 19 trapping sites), Almaty region (surroundings of Tekeli city, Rudniychniy, and Bakanas: four trapping sites) and Almaty city (seven trapping sites) during spring, summer, autumn, and winter seasons (Figure 1 and Supplementary Table S1).

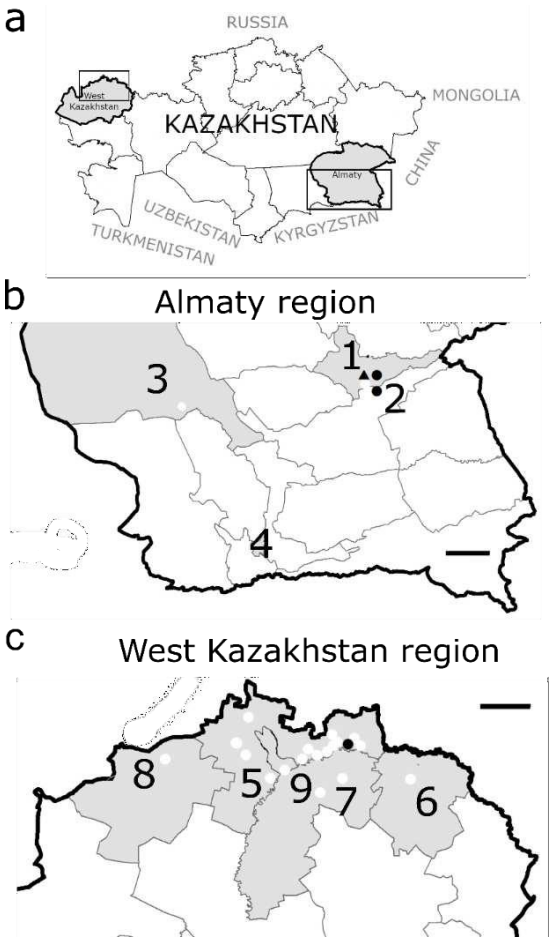

Figure 1. Geographical location of the sampling points for small mammals in Kazakhstan. (**a**): Kazakhstan is divided in 14 oblasts (=regions) and located in Central Asia. (**b**): Almaty region and Almaty city: **1.** Tekeli city: 2 trapping sites; **2.** village Rudniychniy: 1 trapping site; **3.** village Bakanas: 1 trapping site; **4.** Almaty city: 7 trapping sites; (**c**): West Kazakhstan region: **5.** district Bayterek: 12 trapping sites; **6.** district Borili: 1 trapping site; **7.** district Terekti: 2 trapping sites; **8.** district Taskala: 1 trapping site; **9.** Oral city: 3 trapping sites. Sampling locations: white dots. Species and location of infected rodents: ● *Microtus arvalis*, ▲ *Dryomys nitedula*. Black frames = regions magnified in (**b**) and (**c**), size marker = 150 km.

Snap traps were set overnight at 5 m intervals baited with cured pork fat. In the early morning, captured small mammals were collected, stored on dry ice, and transported to the laboratory for immediate processing. After morphological identification of the species,

necropsy was performed, and internal organs (lung, heart, brain, kidney, liver, spleen, ears, and transudate) were aseptically collected and stored in RNA later (Thermo Scientific, Langenselbold, Germany) at −20 °C until further use [27].

2.2. RNA Extraction, PCR Amplification and Sequencing

Lung tissue samples were homogenized in 1 mL MEM for 2 min at 30 Hz in a TissueLyser II (Qiagen, Hilden, Germany). RNA was extracted from 140 µL homogenized supernatant using a commercial QiAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. To determine the sequences of parts of the S and L segments, RNA was reverse-transcribed and amplified using primers detecting a variety of orthohantaviruses and subsequently sequenced using terminator cycle sequencing. In detail, for the S segment, a conventional PCR was applied using Superscript III one step RT-PCR system with Platinum Taq high fidelity polymerase (Invitrogen, Langenselbold, Germany) and the primers DOBV-M6 (5′-AGYCCWGTNATGRGWGTRATTGG-3′) and DOBV-M8 (5′-GAKGCCATRATNGTRTTYCKCATRTCCTG-3′), as described elsewhere [28,29]. The RT-PCR products were analysed using a 1.5% agarose gel with an expected amplicon size of 380 base pairs (bp). To detect a partial L-segment sequence (230 bp), a real-time RT-PCR using a Qiagen One Step RT-PCR mix was performed. Here, the primer-mix contained forward (1a-fw: 5′-TGATGCATATTGTGTGCAGAC-3′, 1b-fw: 5′-TGATGCATACTGTGTGCAAAC-3′, 1c-fw: 5′-CAGTATGATGCATACTGTGTCCAA-3′, 1d-fw: 5′-TGATGCCTATTGTGTTCAGAC-3′) and reverse (1a-rev: 5′-CTTGCTCTGTTTTGAATCTCA-3′, 1b-rev: 5′-CTTGCTCGGTGTTGAATCGCA-3′, 1c-rev: 5′-CCTGTTCTGTATTAAATCTCA-3′, 1d-rev: 5′-CTTGTTCAGTCTTGAATCTCA-3′) (0.125 µM each) primers, complemented with EvaGreen (VWR International, Vienna, Austria) as PCR reagents [30].

To confirm the species determination of the small mammals, a *cytochrome b* (mt-Cytb) gene sequencing was applied as described in [31]. For analysis of the mitochondrially encoded Cytb, supernatant from homogenised rodent lung tissue in elution buffer (Qiagen, Hilden, Germany) was used. A total of 400 ng of DNA were amplified by PCR using the primer combination Cytb-Uni-fw (5′-TCATCMTGATGAAAYTTYGG-3′) and Cytb-Uni-rev (5′-ACTGGYTGDCCBCCRATTCA-3′) targeting an approximately 1000 bp long fragment. The PCR was enabled by using the Invitrogen Platinum Taq High Fidelity DNA Polymerase (ThermoFisher Scientific, Langenselbold, Germany).

All positive PCR products (fragments of the S and L segment, Cytb fragments) were purified using a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced according to the manufacturer's instructions by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Langenselbold, Germany) and a 3730xl DNA Analyzer (Applied Biosystems, Langenselbold, Germany).

2.3. Phylogenetic Analysis

The generated nucleotide sequences were aligned using the ClustalW method in Bioedit 7.2.5. Prior to alignment, the sequences were trimmed for the primers resulting in final sequence lengths of 346 nucleotides (nt) for the S segment and 184 nt for the L segment that were then used for the phylogenetic analysis. Phylogenetic trees were constructed in MEGA X with the Maximum Likelihood method based on the Tamura 3-parameter model [32]. These analyses involved published S and L segment nucleotide sequences from GenBank trimmed to the same length with accession numbers listed in the captions to Figures 2 and 3. To set an outgroup in the phylogenetic trees, sequences of PUUV S and L segments, also trimmed to the respective lengths, were used (NC005224 and NC005225, respectively).

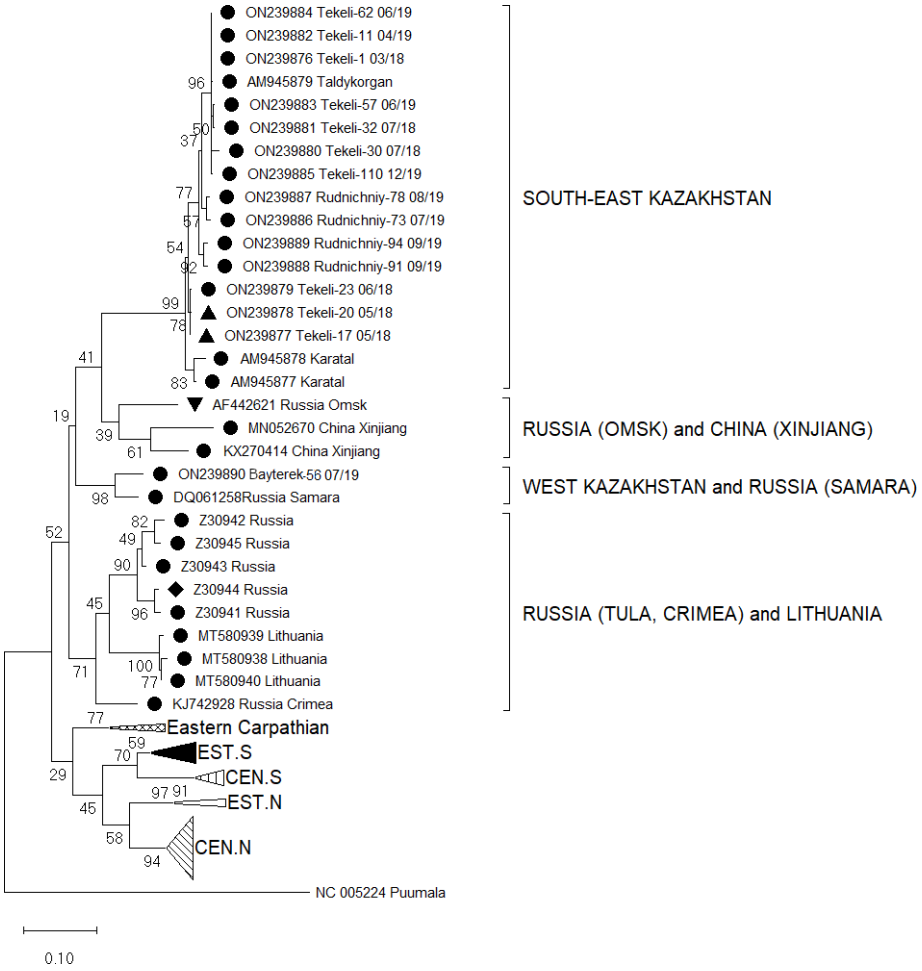

Figure 2. Phylogenetic analysis by Maximum Likelihood method of the S segments (346 nucleotides (nt), positions of sequences 715–1061 nt in regard to the reference sequence AM945879) of Tula virus in Kazakhstan. The tree with the highest log likelihood (−5756.38) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 92 nucleotide sequences: Central North (CEN.N): KU139579, KU139576, KU139577, KU139578, DQ662094, HQ697346, HQ697344, HQ697347, HQ697351, GU300137, GU300136, EU439952, EU439947, EU439949, EU439948, EU439950, EU439946, EU439951, KU139534, KU139535, KU139537, KU139538, KU139598, KU139595, KU139596, KU139599, KU139529, KU139528, KU139531, KU139530, KU139533, DQ662087, DQ768143; Eastern North (EST.N): AF063897, AF289819, AF289820, AF289821; Central South (CEN.S): AF164093, HQ697350, HQ697348, HQ697349, HQ697355, HQ697353, HQ697354, HQ697357; Eastern South (EST.S): AJ223601, U95312, KF184327, KF184328, NC005227, Z69991, Z49915, Z48741, AJ223600, Z48574, KU139560; Eastern Carpathian: AF017659, Y13980, KF557547, Y13979; Russia Tula: Z30941, Z30942, Z30943, Z30944, Z30945; Russia Crimea: KJ742928; Lithuania: MT580938, MT580939, MT580940; Russia Samara: DQ061258; Russia Omsk: AF442621; China Xinjiang: MN052670, KX270414; South-East Kazakhstan: AM945877, AM945878, AM945879, outgroup Puumala NC005224. Host Species: ● *Microtus arvalis*, ▲ *Dryomys nitedula*, ◆ *Microtus rossiaemeridionalis*, ▼ *Microtus gregalis*.

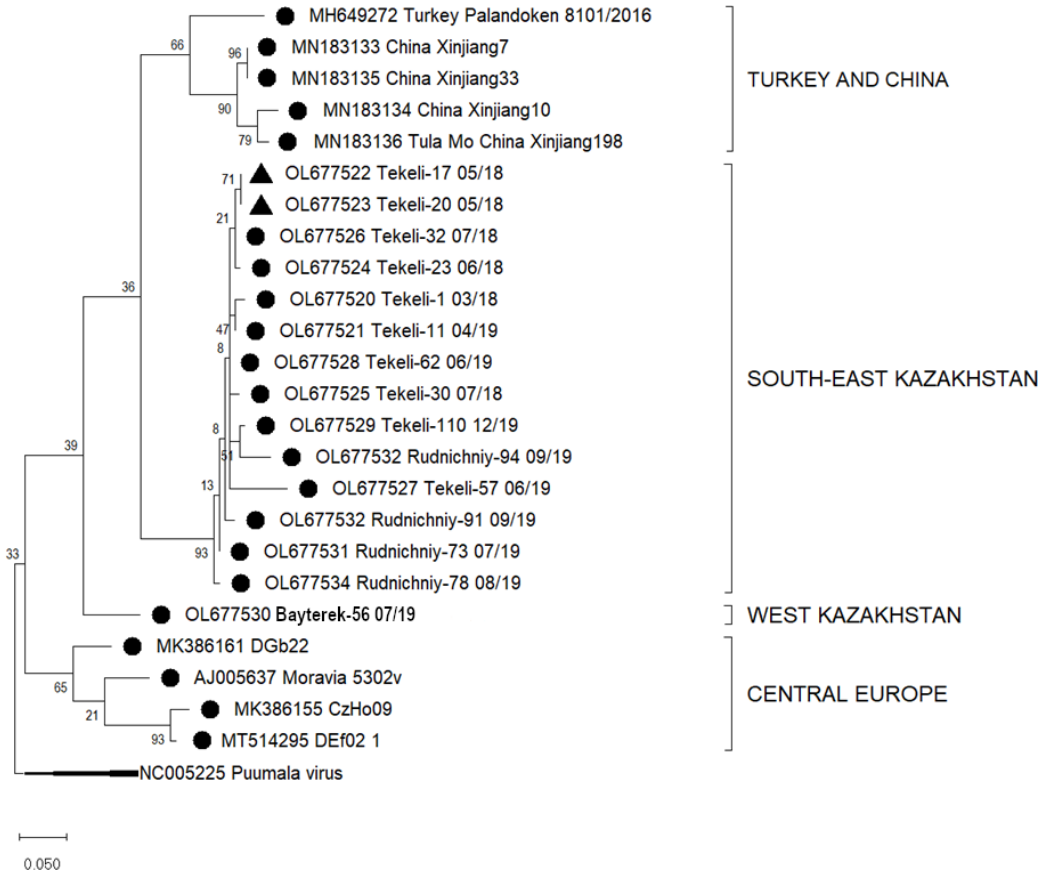

Figure 3. Phylogenetic analysis by Maximum Likelihood method of the L segments (184 nucleotides (nt), positions of sequences 5187–5371 nt in regard to the reference sequence NC005226) of Tula virus in Kazakhstan. The tree with the highest log likelihood (−1345.67) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 25 nucleotide sequences: Turkey: MH649272; China: MN183133, MN183135, MN183134, MN183136; Europe: AJ005637, MK386161, MK386155, MT514295, outgroup Puumala NC005225. Host Species: ● *Microtus arvalis*, ▲ *Dryomys nitedula.*

3. Results

In total, 621 small mammals were collected in nine sampling areas, at all together 30 trapping sites during the years of 2018–2019 (Table 1).

Table 1. All species captured in snap traps in the sampling areas of interest.

Small Mammal Species	West Kazakhstan (19 Trapping Sites)	Almaty Region (4 Trapping Sites)	Almaty City (7 Trapping Sites)
Microtus arvalis (Common vole)	13	72	1
Myodes glareolus (Bank vole)	12	0	0
Microtus kirgisorum (Tien Shan vole)	0	0	49
Apodemus uralensis (Ural or Pygmy field mouse)	128	84	47
Mus musculus (House mouse)	62	27	39
Rattus norvegicus (Brown rat)	0	0	39

Meriones meridianus (Midday jird)	0	2	0
Dryomys nitedula (Forest dormouse)	2	13	0
Sorex araneus (Common shrew)	1	0	0
Sorex minutus (Eurasian pygmy shrew)	0	1	1
Crocidura suaveolens (Lesser white-toothed shrew)	0	0	28
Total	**218**	**199**	**204**

These small mammals represent eleven species from four families: Cricetidae (*M. arvalis*, *M. glareolus*, *M. kirgisorum*), Muridae (*A. uralensis*, *M. musculus*, *R. norvegicus*, *M. meridianus*), Gliridae (*D. nitedula*) and Soricidae (*S. araneus*, *S. minutus*, *C. suaveolens*). Sex distribution of collected mammals was almost equal with 59% male and 41% female.

Out of all 621 collected small mammals 15 (2.4%) were positive for orthohantavirus RNA (Supplementary Table 1). In Almaty city, all analysed rodents failed to yield a positive result. The infected individuals represented two species, *M. arvalis* (*n* = 13, 15.1%) and *D. nitedula* (*n* = 2, 13.3%) (Table 2). Three *M. arvalis* and both of the orthohantavirus carrying *D. nitedula* samples were further tested by *cytochrome b* gene-specific PCR and subsequent sequence analysis [31] to confirm the morphological determination. The Cytb sequence of Tekeli23 *M. arvalis* (ON513439) was 99% similar to a nucleotide sequence of *M. arvalis* originating from Russia, Ekaterinburg (MG703092). Both the *D. nitedula* Tekeli17 (ON513437) and Tekeli20 (ON513438) species were also confirmed by mitochondrial *cytochrome b* sequencing. The two sequences are 98% identical to a sequence from *D. nitedula* described from Mongolia (LR131101). All orthohantavirus infected specimens where either adults (*n* = 11) or sub-adults (*n* = 4).

Table 2. Result of the molecular biological screen for orthohantavirus RNA among small mammals captured in the regions of interest.

Small Mammal Species	Total Collected	Sex Ratio Male/Female	Number of Positive Samples (Male/Female)	Percentage of Positive Samples [%]
Microtus arvalis	86	40/46	13 (8/5)	15.1
Dryomys nitedula	15	7/8	2 (1/1)	13.3
Myodes glareolus	12	11/1	0	0
Microtus kirgisorum	49	26/23	0	0
Apodemus uralensis	259	163/96	0	0
Mus musculus	128	83/45	0	0
Rattus norvegicus	39	16/23	0	0
Meriones meridianus	2	2/0	0	0
Sorex araneus	1	0/1	0	0
Sorex minutus	2	1/1	0	0
Crocidura suaveolens	28	15/13	0	0
Total	**621**	**364/257**	**15 (9/6)**	**2.4**

A partial S segment sequence analysis revealed that all 15 small mammals harboured RNA of TULV. The obtained sequences were aligned with published TULV partial S segments available for Central Asia, Eastern and Central Europe, and China. These included clades from different geographic regions such as Central North (CEN.N), Eastern North (EST.N), Central South (CEN.S), Eastern South (EST.S), Eastern Carpathian,

Russia (Tula, Crimea, Samara, and Omsk), Lithuania, and China (Xinjiang) (Figure 2). A nucleotide sequence identity matrix of the detected S segments compared with sequences of geographically relevant regions reveals that the sequences have an identity range from 78.9–100% (Table 3).

Table 3. Nucleotide sequence identity of the partial Tula virus (TULV) S-segments detected from Kazakhstan in comparison with published sequences from other Eurasian regions (%).

S Segment Cluster	South-East Kazakhstan	China (Xinjiang)/ Russia (Siberia)	Russia (Tula and Crimea)	West Kazakhstan	Russia (Samara)
South-East Kazakhstan	94.3–100	78.9–99.4	78.9–99.4	78.9–99.4	75.8–99.1
China (Xinjiang)/ Russia (Siberia)		84.5–87.5	81.6–98.5	82.1–87.5	79.9–88.9
Russia (Tula and Crimea)			87.5–98.5	84.5–98.5	85.6–97.9
West Kazakhstan				100	93.4
Russia (Samara)					100

By comparing the newly identified TULV sequences with published genomes, four clusters can be classified that are geographically relevant for Kazakhstan (Figure 2): (I) The South-East Kazakhstan cluster consists of new virus sequences from Tekeli and Rudnichniy and already published sequences from Taldykorgan (AM945879) and Karatal (AM945877, AM945878) with a nucleotide sequence identity range of 94.3–100%. (II) The second neighbouring cluster from China and Russia includes sequences from Xinjiang (KX270414, MN052670) and from Omsk in Russian Siberia (AF442621) with a nucleotide sequence identity ranging from 84.5–87.5% within the cluster. (III) The third cluster are sequences from the Tula area of Russia (Z30941-4) and from Crimea (KJ742928) with an identity range of 87.5–98.5%. (IV) One positive sample (*M. arvalis*, Bayterek-56 07/19) from West Kazakhstan had a 93.4% sequence identity with the Samara virus from Russia (DQ061258). These two virus sequences form a separate cluster from all the other sequenced viruses (Figure 2).

A 78.9–99.4% nucleotide sequence identity is noticeable between the cluster of southeast Kazakhstan (I) that contains genomes form China and Siberia (II), as well as among the clade of Tula and Crimea area of Russia (III) and with the new sequence from West Kazakhstan (IV). The sequences from southeast Kazakhstan (I) are 75.8–99.1% similar to the Samara virus of Russia (IV).

The sequences from West Kazakhstan have 84.5–98.5% identity with variants from the Tula region and Crimea (III) and 82.1–87.5% identity with genomes from China and Siberia (II), respectively.

In silico translated S segment sequences of all TULV sequences included in this study showed 86–100% amino acid sequence identity for the N protein to other variants (Supplementary Figure S1).

Similarly, the sequences of parts of the L segment from Almaty and West Kazakhstan regions were aligned with other L segment sequences available from GenBank. These resulted in four clusters of TULV from various geographic locations. Sequences of the 14 samples from Almaty region grouped in one subcluster (South-East Kazakhstan, I), sequences from China (Xinjiang, MN183133-6) and Turkey (Palandoken, MH649272) in a second cluster (II). These sequences show nucleotide sequence similarities of 80–99.3%. One sample from West Kazakhstan (Bayterek-56 07/19, *M. arvalis*, III) grouped distant from the other sequences (Figure 3) and had a nucleotide sequence similarity of 80.6–99.3% to the samples from South-East Kazakhstan (I) (Table 4).

Table 4. Nucleotide sequence identity of the partial Tula virus (TULV) L segment sequences in Kazakhstan and other Eurasian regions (%).

L Segment Cluster	Turkey and China	South-East Kazakhstan	West Kazakhstan	Central Europe
Turkey and China	85.9–100	80–99.3	81.6–85.9	78.3–97.2
South-East Kazakhstan		89.3–100	80.6–99.3	76.9–88.3
West Kazakhstan			100	79.4–97.2
Central Europe				87–97.2

By translating these nucleotide sequences into its short peptide sequence of 61 amino acids, two recurring substitutions become apparent. The sequences Tekeli-110 (OL677529) and Rudnichniy-94 (OL677532) show at position 1760 a P versus R exchange and at position 1773 a K versus E aberration in comparison to published consensus sequences (Supplemental Figure S2).

4. Discussion

We designed a study to screen for orthohantavirus RNA in small mammals in the Republic of Kazakhstan regions West Kazakhstan, Almaty region, and Almaty city. Here, we demonstrate for the first time the presence of TULV in West Kazakhstan and confirm it in the Almaty region in Kazakhstan. The rate of positive individuals of *M. arvalis* is 15.1% (13/86), which agrees with previous studies [33,34]. Among all positive samples, males accounted for 60% (n = 9), which is consistent with other studies showing that male small mammals have a greater infection rate for orthohantaviruses (Table 2) [35].

West Kazakhstan is the only official orthohantavirus endemic region with registered human cases of orthohantaviruses infections so far [36,37]. Long-term investigations of host reservoirs starting from 2001 by colleagues from the Oral antiplague station revealed natural foci of orthohantaviruses in the floodplains of the Ural River. This area directly borders the Russian Orenburg and Samara regions, where orthohantavirus is also endemic [26,38]. Several small mammals that are also spread in this region such as *M. glareolus*, *M. arvalis*, *A. uralensis*, and *M. musculus* contained orthohantavirus antigens [26]. Our study could confirm the existence of TULV in West Kazakhstan region in *M. arvalis*, but only in one specimen. Actually, we expected to find the presence of PUUV, due to clinical manifestations of hospitalized patients with HFRS that is primarily caused by PUUV. Additionally, *M. glareolus,* the main host reservoir of PUUV is very common in this region. However, the number of captured *M. glareolus* and other small mammals was rather low to draw a statistically convincing picture on the spread of orthohantavirus in this area. Still, this study is the first to perform molecular-biological methods in the region of West Kazakhstan and generated the first orthohantavirus sequence from TULV [26,37].

In this study, for the first time, small mammals were screened for the presence of orthohantaviruses in Almaty city, but no positive results were revealed in the captured rodent species that were *M. kirgisorum*, *A. uralensis*, *R. norvegicus,* and *M. musculus*. The latter where the most captured animals in Almaty in this study. All these species might carry different orthohantaviruses such as, e.g., SEOV, but the primers used in this study are detecting all species of orthohantaviruses as shown in an internal validation of the primer sets for certified diagnostics [39]. The reason why there were no traces of orthohantavirus detected in the city are manifold but may rest in the different living conditions and species composition of the rodent population. However, as PUUV-reactive antibodies were found in a retrospective study in patients with fever of unknown origin [23], further studies have to be conducted in different geographic areas of Almaty city in order to unveil the real prevalence in the city.

Nevertheless, in the Almaty region, an area stretching north of Almaty city, TULV was identified and sequenced in several specimens captured in Tekeli city and Rudnichniy village. All TULV RNA was detected in two different species of small mammals, *M. arvalis* and *D. nitedula*. *M. arvalis* is a commonly known host for TULV.

Interestingly, however, we also found TULV in *D. nitedula* of the Gliridae family that represents a novel host species for TULV. A cytochrome b sequence analysis confirmed the species. So far, the literature only reports on TULV in species belonging to the Arvicolinae subfamily, such as *Microtus* spp. and *Lagurus lagurus* [40,41]. However, by comparing the capture sites of those two infected specimens, it becomes apparent that the spots in Tekeli had a spatial distance of only 325 m. In this region, *D. nitedula* is a common mammal, mostly living on trees but also reported to hunt for edibles on the ground, since also the traps were only located on terra firma. There, it may have indirect contact with *M. arvalis* that builds nests in subterraneous burrows but also gathers edibles on the ground. The infection of atypical host species with orthohantavirus is designated as a spill-over infection and is reported in high incidence areas in Europe [41]. Since we identified several infected rodents in the Tekeli area and the S segment sequences derived from *D. nitedula* and *M. arvalis* are almost identical, such a spill-over event is in the scope of possibilities [42–44]. Nevertheless, this result implies the need for a more extensive follow-up host-study for infected small mammals in the area of the Almaty region to obtain information on the actual distribution of orthohantaviruses in this area.

To further estimate the connection of these viruses, we performed sequencing of parts of the S and L segments. Sequence similarities for the partial S segments of the clusters of South-East Kazakhstan (I) and West Kazakhstan/Samara (IV) resemble these of previous studies [41]. Furthermore, the phylogenetic analysis of the partial S segment sequences enabled the classification of TULV in a broad geographical range [43,45,46]. Our results highlight that TULV from West Kazakhstan is indeed in close evolutionary relationship with TULV described in Samara, the adjoining region in the Russian Federation (DQ061258). Almaty region (Tekeli and Rudnichniy) has its own cluster separated from all other TULV sequences for the S segment (Figure 2). Additionally, it is evident that the West Kazakhstan TULV S segment sequence is only distantly related to other Kazakhstan sequences as, for instance, from the Almaty region, a region over 2000 km apart from West Kazakhstan. Sequences from the Tekeli city and Rudnichniy village in the Almaty region shared a close relationship with previously published sequences of *M. arvalis* sampled in the village of Karatal and Taldykorgan city, located also in the Almaty region [22]. It is highly probably that there exist different geographic lineages of TULV in Kazakhstan transmitted by different lineages of rodents as recently shown for TULV sequences in Europe [33,40,41].

The sequence relationships identified for the S sequence analysis can also be identified in the analysis of the partial L segment sequences, where we could show that the TULV L segment sequence from West Kazakhstan region formed its own distinct geographic cluster. In general, published sequences for the L segment in this region are sparse and for the Almaty region, we describe for the first time also TULV L segment sequences, in comparison to a previous study that only analysed the S segment [22]. Sequences from Tekeli and Rudnichniy in Almaty region cluster in an individual branch in one big cluster with sequences from China and Turkey (Figure 3) [47,48]. This finding goes along with previous studies who have illustrated that genetic clustering of TULV is largely according to geographic regions [22,33].

5. Conclusions

Here, we screened 621 small mammals for their orthohantavirus infection rate. Interestingly, we only identified the relatively benign TULV species, a finding that is contrary to the expectation risen by patients with episodes of haemorrhagic fever in Kazakhstan hospitals. Knowledge on the pathogenicity of TULV and the impact of TULV-associated disease in humans is limited. Only few cases, mostly mild, were described in Europe, some of them in immunocompromised patients [10–13,49]. In certain risk groups, e.g., forest workers, a higher antibody prevalence against TULV was found in comparison to the normal population [11,12]. However, the severe cases of HFRS observed in the

hospitals in West Kazakhstan are most probably not induced by an infection with TULV but rather by PUUV. The exact endemic areas for this virus in Kazakhstan remain obscure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14061258/s1, Figure S1: All available amino acid S segment sequences from Kazakhstan and close geographic regions in Russia (Omsk, Samara) and China; Figure S2: All available amino acid L segment sequences from Kazakhstan and close geographic regions in Russia (Omsk, Samara) and China; Table S1: Detailed information on trapping sites of small mammals.

Author Contributions: N.T. (Nur Tukhanova), S.F., L.P., E.W., and S.E. conceived the layout of the project. N.T. (Nur Tukhanova), E.W., V.S., A.B., N.M., A.Z., and I.L. participated in the fieldwork and the preparation and analysis of collected small mammals. I.L. performed morphological determination of the captured small mammals. N.T. (Nur Tukhanova), A.S. (Anna Shin), E.W., N.T. (Nurkeldi Turebekov), K.A., and T.N. contributed tissue homogenization and RNA extraction of collected samples. N.T. (Nur Tukhanova) performed molecular biology testing and analysis. A.S. (Alexandr Shevtsov) was in charge of the sequencing. N.T. (Nur Tukhanova) wrote the draft manuscript. N.T. (Nur Tukhanova) and L.P. created the figures and tables. T.Y., G.T., L.Y., G.F., M.H., and S.F. contributed additional information and reviewed the manuscript. S.E. and L.P. supervised the project. L.P. was in charge of the revision process. All authors have read and agreed to the published version of the manuscript.

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