INVESTIGATION OF ZOONOTIC PATHOGENS IN CENTRAL ASIA

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1 Abbreviation list

APS	anti-plague station
BSL	biosafety level
C	capsid
CCHF	Crimean Congo haemorrhagic fever
CCHFV	Crimean Congo haemorrhagic fever virus
CFR	case fatality rate
CNS	central nervous system
CRL	Central Reference Laboratory
CSF	cerebrospinal fluid
DENV	Dengue virus
DNA	desoxyribonucleic acid
DOBV	Dobrava orthohantavirus
E	envelope
EBOV	genus *Ebola virus*
EDP	extremely dangerous pathogens
ELISA	enzyme-linked immunosorbent assay
FUO	fever of unknown origin
FWS	fever without source
HCPS	Hantavirus cardiopulmonary syndrome
HF	haemorrhagic fever
HFRS	haemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HNTV	Hantaan orthohantavirus
HPS	Hantavirus pulmonary syndrome
Ig	Immunoglobulin
IIFA	indirect immunofluorescence assay
JEV	Japanese encephalitis virus
LASV	Lassa mammarena virus
M	membrane
MARV	genus *Marburg virus*
MERS-CoV	Middle East respiratory syndrome-related coronavirus
MLST	multilocus sequencing typing
MUO	meningitis of unknown origin
MVEV	Murray Valley fever virus
NCBI	National Center for Biotechnology Information
NE	*nephropathia epidemica*
NS	non-structural
OHF	Omsk haemorrhagic fever
OHFV	Omsk haemorrhagic fever virus
ORF	open reading frame
PCR	polymerase-chain-reaction
PPE	personal protective equipment

pr	precursor
PUUV	Puumala orthohantavirus
RMSF	Rocky Mountain spotted fever
RNA	ribonucleic acid
RT	reverse transcriptase
RVFV	Rift Valley fever virus
SARS-CoV-2	severe acute respiratory syndrome coronavirus type 2
SEOV	Seoul orthohantavirus
SFG	spotted fever group
SPC SEEM	Scientific and Practical Center of Sanitary and Epidemiological Expertise and Monitoring
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TBEV-Eu	Tick-borne encephalitis virus – Eurasian subtype
TBEV-FE	Tick-borne encephalitis virus – Far-Eastern subtype
TBEV-Sib	Tick-borne encephalitis virus – Siberian subtype
TG	typhus group
TULV	Tula orthohantavirus
VHF	viral haemorrhagic fever
WHO	Word Health Organisation
WNV	West Nile virus
YFV	Yellow fever virus

2 Summary

Kazakhstan ranks ninth in the world in terms of country size. With its varied landscapes and hot and dry climate, it is a perfect home for many naturally occurring dangerous infectious diseases. Many of them are not well studied and due to the lack of contemporary diagnostic no or only poor differential diagnostics can be performed.

Currently in Kazakhstan, most infectious diseases are diagnosed by physicians on an analysis of the clinical picture. Only in officially endemic areas for certain pathogens, physicians might be aware of the possibility of an infection with a pathogen, and only in these areas cases are publicly recorded. This system implies that there is a lack of reliable clinical laboratory diagnostics, trained laboratory staff and well-equipped laboratories all over Kazakhstan. In many cases, the aetiological agent cannot be determined, and patients with only having a fever, are diagnosed with fever of unknown origin. If patients' symptoms also include headache, their diagnosis is suspected meningitis. In addition, the prevalence of many zoonoses is not well investigated in humans, hosts (like rodents) and vectors (like ticks).

To address this issue, we investigated several viral and bacterial zoonotic pathogens in humans, rodents and ticks in areas of Kazakhstan that are not yet officially endemic for specific pathogens. The studies included viral pathogens belonging to the family of *Flaviviridae* like Omsk haemorrhagic fever virus (OHFV), Tick-borne encephalitis virus (TBEV) and West Nile virus (WNV). Also, *Orthohantavirus* and the bacterial genus of *Rickettsia* were investigated. The investigations were performed in human samples for OHFV, TBEV and WNV, in biopsies from small mammals for OHFV, *Orthohantavirus, Rickettsia* spp. and ticks for OHFV in several regions of Kazakhstan using enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IIFT) as well as real-time and conventional polymerase chain reaction (PCR). All mentioned methods had to be implemented in different laboratories in Kazakhstan.

In a study of sera and cerebrospinal fluid (CSF) from patients with the suspected meningitis, the symptoms were caused by agents such as TBEV, WNV and OHFV in 6% of the patients examined. In addition, it was possible for the first time to detect OHFV beyond Russia in several regions of Kazakhstan in patients, but also in rodents and ticks. Further biosurveillance of small mammals and ticks also shows that pathogens such as OHFV, *Orthohantavirus* and *Rickettsia* spp. are wider spread than

currently assumed by the Kazakhstan authorities. For *Orthohantavirus* a phylogenetic analysis from several geographical regions was first performed in Kazakhstan. *Rickettsia*, a bacterial zoonotic agent, was also investigated in rodents for the first time in this country. The studies were conducted to complete a series of investigations related to the occurrence and distribution of circulating strains of *Rickettsia* spp. in humans, ticks and rodents in Kazakhstan. This study showed, that species of genus *Rickettsia* are not only detectable in rodents, but small vertebrates may also play a role in the *Rickettsia* life cycle.

All these studies have been performed in Kazakhstan, that functions here as a representative of a Central Asian country. It is to be assumed, that the situation is similar in other neighbouring former Soviet states in the region. To solve public health issues according to missing reliable diagnostics, trained laboratory personal, sensitized physicians, funding has to be secured to implement modern laboratories all over the country and in training of medical and laboratory stuff. In addition, it is necessary to intensify the monitoring of the spread of different zoonotic agents in different hosts.

3 Zusammenfassung

Kasachstan ist das neuntgrößte Land der Erde. Mit seiner großen landschaftlichen Vielfalt und seinem heißen und trockenen Klima ist es ein idealer Nährboden für viele natürlich vorkommende gefährliche Infektionskrankheiten. Viele von ihnen sind in diesem zentral asiatischen Staat kaum erforscht und können aufgrund fehlender moderner molekularbiologischer Diagnostikmethoden nur unzureichend diagnostiziert werden. Die Infektionskrankheiten stellen eine erhebliche Gefahr für Mensch und Tier in Kasachstan dar.

Aktuell werden in Kasachstan die meisten Infektionskrankheiten von Ärzten durch die Feststellung von Symptomen, also aufgrund des klinischen Bildes, diagnostiziert. Ausschließlich in Regierungsbezirken, welche von der Regierung offiziell als endemische Gebiete für bestimmte Krankheitserreger ausgewiesen sind, sind die Ärzte von der Möglichkeit einer Infektion mit einem Erreger sensibilisiert und folglich werden solche Infektionsfälle behördlich erfasst. Diese Herangehensweise impliziert, dass es in dem ganzen Land an zuverlässiger klinischer Labordiagnostik, geschultem Laborpersonal und gut ausgestatteten Laboratorien mangelt. In vielen Krankheitsfällen kann der Erreger nicht bestimmt werden und bei Patienten, die als einziges Symptom Fieber aufweisen, wird Fieber unbekannter Genese diagnostiziert. Leiden die Patienten zudem an Kopf- und Nackenschmerzen, besteht der Verdacht auf eine Meningitis mit unklarer Genese. Neben der fehlenden Diagnostik am Menschen ist zu der Prävalenz der Zoonotischen Pathogene bei den Wirten (z.B. Nagetiere) und den Vektoren (Zecken) in Kasachstan wenig publiziert.

Um umfangreiche Informationen zur Prävalenz in verschiedenen Wirten (Menschen, kleinen Säugetieren und den Zecken, welche als Vektoren dienen) zu erhalten, wurden hier unterschiedliche virale und bakterielle Zoonoseerreger in verschiedenen Wirten und Vektoren in Gebieten Kasachstans untersucht, welche noch nicht offiziell als endemisch für bestimmte Erreger gelten. Die Studien umfassten virale Erreger aus der Familie der *Flaviviridae*, wie das Omsk-Hämorrhagische-Fieber-Virus (OHFV), das Zeckenenzephalitis-Virus (TBEV) und das West-Nil-Virus (WNV). Auch das *Orthohantavirus* und die Bakteriengattung der *Rickettsien* wurden untersucht. Die Untersuchungen wurden an humanen Liquor und Blutserum Proben (bezüglich OHFV, TBEV und WNV), an Biopsien von Kleinsäugern (im Hinblick auf OHFV, *Orthohantavirus*, *Rickettsia* spp.) und Zecken (in Bezug auf OHFV) in verschiedenen

Regionen Kasachstans mittels *enzyme-linked immunosorbent assay* (ELISA) und *indirect immunofluorescence assay* (IIFT), *real-time*- und konventioneller PCR durchgeführt. Alle genannten methodischen Ansätze mussten zunächst in mehreren Laboratorien vor Ort etabliert werden.

In einer Studie an Seren und Liquor von Patienten mit Verdacht auf Meningitis wurden bei 6% der untersuchten Patienten die Symptome durch Erreger wie TBEV, WNV und OHFV verursacht. Außerdem konnte OHFV erstmals über Russland hinaus in mehreren Regionen Kasachstans bei Patienten, aber auch bei Nagetieren und Vektoren nachgewiesen werden. Weitere Überwachungsstudien von Kleinsäugern und Zecken zeigen, dass die Erreger wie OHFV, *Orthohantavirus* und *Rickettsia* spp. weiterverbreitet sind als von den kasachischen Behörden derzeit angenommen. Zudem wurde für *Orthohantavirus* erstmals in Kasachstan eine phylogenetische Analyse aus verschiedenen geografischen Regionen durchgeführt.

Rickettsia, ein bakterieller Zoonoseerreger, wurde in Kasachstan zum ersten Mal auch bei Nagetieren untersucht, um eine Reihe von vorangegangenen Untersuchungen über das Ausmaß der zirkulierenden Stämme von *Rickettsia* spp. bei Menschen, Zecken und Nagetieren in Kasachstan zu vervollständigen. Diese Studie zeigte, dass mehrere Arten der Gattung *Rickettsia* auch in Nagetieren nachweisbar sind und dass kleine Wirbeltiere möglicherweise eine Rolle im Lebenszyklus der Rickettsien spielen.

Da Kasachstan als Vertreter eines zentralasiatischen Landes angesehen werden kann, ist davon auszugehen, dass die Situation in benachbarten ehemaligen Sowjetstaaten der Region ähnlich ist. Um die Probleme des öffentlichen Gesundheitswesens entsprechend der fehlenden zuverlässigen Diagnostik, des geschulten Laborpersonals und der sensibilisierten Ärzte zu lösen, müssten die finanziellen Mittel für die Einrichtung moderner Laboratorien in ganz Kasachstan und für die Ausbildung von medizinischem und Laborpersonal langfristig gesichert werden. Darüber hinaus müsste die Überwachung der Verbreitung der unterschiedlichen zoonotischen Erreger in unterschiedlichen Wirten intensiviert werden.

4 Introduction

4.1 Zoonoses and zoonotic agents

A zoonosis or zoonotic disease is an infectious disease originating from a pathogenic agent (*e.g.,* bacterium, virus, parasite or prion) that is passed from an arthropod (ticks, mosquitos and fleas) or vertebrate *(e.g.*, birds, rodent and small mammals) to a human. Person-to-person transmission is very common and increases the infection rate. The zoonotic agents are common in both humans and animals and can be transmitted from animals to humans and *vice versa*. The ways of transmission are either by direct contact with infected animals, such as bite injuries or smear infections, by the consumption of contaminated water or by consumption of animal-derived food like eggs, milk, and meat from infected animals. Furthermore, the inhalation of contaminated aerosols plays an important role. Zoonotic agents can also be transmitted by intermediate vectors such as mosquitos and ticks (Cavalerie *et al.*, 2021; Cross *et al.*, 2019; Hubálek, 2003).
The zoonotic agents can be viral (*e.g.* Chikungunya virus, Tick-borne encephalitis virus, a member of the genus *Orthohantavirus*, *Marburg virus* (MARV)) (Kallio-Kokko *et al.*, 2005; Trovato *et al.*, 2020), bacterial (*e.g.* Bacillus anthracis, Escherichia coli, *Rickettsia* spp. or Francisella tularensis) (Chikeka and Dumler, 2015), parasites (*e.g. Plasmodium* spp*., Leishmania* spp., *Trichinella* spp.), (Thompson, 2013) or also prions leading to Creutzfeldt-Jakob-Disease (Barria *et al.*, 2014).
Zoonotic diseases are a problem to healthcare systems all over the world, especially in underdeveloped countries. Generally, the incidence and prevalence of most zoonoses is difficult to estimate. Many zoonotic infections remain undiagnosed because the human symptoms are too mild, furthermore there is no obligation to report most zoonoses. Nonetheless, the more frequent and the more direct the contact with animals, the greater the risk of being infected with a zoonosis.
Global health depends on a large network of diverse human, animal and environmental factors and interactions, which are summarised by the World Health Organisation (WHO) under the term "One Health" (Thompson, 2013). Worldwide, 75% of the emerging human infectious diseases (approx. 1,400) are of zoonotic origin (Cross *et al.*, 2019; Mrzljak *et al.*, 2020; Shaheen, 2022) and can be particularly dangerous to humans. According to the WHO, members of this heterogeneous group of these so-called extremely dangerous pathogens (EDP) are pathogenic species like Bacillus

anthracis, *Borellia* spp*.,* Francisella tularensis*, Legionella* spp*.,* Yersinia pestis*, Filoviruses* (*Ebola virus (EBOV)* and *MARV*), Lassa mammarena virus (LASV), Middle East respiratory syndrome-related coronavirus (MERS-CoV), Monkeypox virus, Nipah virus, Rift Valley fever virus (RVFV) or Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). EDPs are an constant threat to the health of the world population and pose a major challenge to the global health system (WHO, 2022).
The present work “Investigation of zoonotic pathogens in Central Asia” will focus on several viral zoonotic agents such as Omsk haemorrhagic fever virus (OHFV), TBEV, West Nile Virus (WNV) and members of the genus *Orthohantavirus* as well as bacterial zoonotic agents of the genus *Rickettsia.*

4.2 Clinical relevance of zoonotic infections

4.2.1 Fever of unknown origin

In humans, an infection with zoonotic agents can lead to different clinical courses – from mild to life-threatening (Cleri *et al.*, 2007; Trovato *et al.*, 2020). In very weak and mild forms, the clinical picture can show non-specific symptoms, with mild to high continuous fever. If the fever is high, lasts for several days and the aetiologic agent is not known and cannot be detected, it is declared as fever of unknown origin (FUO) or fever without source (FWS).
FUO was first defined by Peterson and Beeson in 1961, indicating a temperature over 38.3°C over a period of more than three weeks without being able to identify the cause after one week of investigation (Petersdorf and Beeson, 1961). In this description, acute, self-limiting fever and healthy humans with chronically elevated temperature are excluded. The clinical picture of FUO was updated in 1991 by Durack and Street and further re-classified into four distinct groups: (1) classical FUO, (2) nosocomial (health-care-associated) FUO, (3) neutropenic (immune-deficient) FUO and (4) human immunodeficiency virus (HIV) - related FUO. According to them, FUO is fever with an undefined diagnosis after three days of hospitalization or after two hospitalizations due to the same symptoms (Durack and Street, 1991). For physicians trying to find its cause, FUO still represents one of the most challenging clinical pictures. The precise incidence and prevalence of FUO remains unknown (Bosilkovski, 2020). Classical FUO can be caused by viral infections, such as Influenza viruses, Rhinoviruses, Parainfluenza viruses (El-Radhi, 2019), but also by Epstein-Barr virus and members

of the genus *Orthohantavirus* (Wright and Auwaerter, 2020). If patients with fever also show symptoms like neck pain and headache, they are diagnosed with meningitis of unknown origin (MUO) (Coates and Jeffery, 2014; Shin *et al.*, 2022).

4.2.2 Viral haemorrhagic fever

Infections with certain virus species form additionally to fever the symptom of haemorrhages. This clinical picture is then called a viral haemorrhagic fever (VHF) (Racsa *et al.*, 2016). Viral haemorrhagic fevers are severe febrile illnesses and are characterized by symptoms such as high fever, malaise, headache, vascular permeability, a decreased plasma volume, coagulation abnormalities, and haemorrhages in different forms (Marty *et al.*, 2006; Racsa *et al.*, 2016). The viruses attack the vascular system and lead to bleeding and a leakage into the tissues surrounding the blood vessels, where it can also be seen visually by the formation of large red areas. Haemorrhages can occur internally, externally but also in a body orifice such as the mouth, nose, ear, urinary tract, vagina or anus. Depending on their position, they can for example be called hematoma, which is a bleeding under the skin, petechial bleeding, which are small red, brown or purple spots on the skin, or subconjunctival haemorrhages, which are damaged blood vessels in the eye. In particular some species of viral families, including *Arenaviridae, Bunyavirdae, Filoviridae, and Flaviviridae*, have been reported to cause haemorrhages along with fever during the course of the disease in humans (Table 1) (Iannetta *et al.*, 2019; Messaoudi and Basler, 2016; Racsa *et al.*, 2016).

Table 1 Overview of the four families and virus species leading to viral haemorrhagic fevers, including information on preventive vaccine available worldwide. HF = haemorrhagic fever, BSL = Biosafety Level according to Germany, CFR = Case Fatality Rate.

Species	Disease	BSL	Host	Vector	Distri-bution	CFR	Vaccine	Treat-ment	Ref-erence
Family Arenaviridae									
Lassa mammarena virus (LASV)	Lassa fever	4	rodents	no	West Africa	10-20%	none	supportive, ribavirin	(Basler, 2019; Iannetta *et al.*, 2019; Marty *et al.*, 2006; Paessler and Walker, 2012; Trovato *et al.*, 2020)
Junin virus	Argentine HF				South America	20-30%	vaccine only in Argentina	supportive, ribavirin, immune plasma	
Machupo virus	Bolivian HF					5-30%	vaccine	supportive, ribavirin	
Sabia virus	Brazilian HF					up to 30%	none		
Guanarito virus	Vene-zuelan HF						none		
Family *Bunyaviridae*									
Crimean-Congo HF (CCHF) virus	CCHF	4	ticks, livestock	ticks	Africa, Central Asia, South-east Europe, Middle East, Balkans	5-30%	vaccine only in Bulgaria	supportive, ribavirin	(Basler, 2019; Brocato and Hooper, 2019; Hooper and Li, 2001; Iannetta *et al.*, 2019; Johnson *et al.*, 2018; Mansfield *et al.*, 2015; Marty *et al.*, 2006; Paessler and Walker, 2012; Schmaljohn and Hjelle, 1997; Schmaljohn, 2012; Trovato *et al.*, 2020)
genus *Orthohanta-virus*: Hantaan (HNTV)-, Seoul (SEOV)-, Dobrava (DOBV)-, Puumala (PUUV) orthohanta-virus	HF with renal Syndrome (HFRS)	3	rodents	no	worldwide	HNTV: 10-15% DOBV: 10-12% SEOV: 1-5% PUUV: <1%	several commercial-ly available vaccines in China, Korea, North-Korea	supportive, ribavirin	
Rift Valley fever (RVF) virus	RVF	3	livestock	mos-quitos	Africa	with HF: 50% without: 1%	only livestock vaccines	supportive, ribavirin	

Family *Filoviridae*									
genus *Ebolavirus* (EBOV)	Ebola	4	bats (?)	no	Africa	50-80%	none	supportive, rNAPc2(?)	(Basler, 2019; Janik *et al.*, 2020; Marty *et al.*, 2006; Paessler and Walker, 2012; Suschak and Schmaljohn, 2019; Trovato *et al.*, 2020)
genus *Marburg-virus* (MARV)	Marburg						none	supportive	
Family *Flaviviridae*									
Dengue virus (1-4) (DENV)	Dengue, Type 1-4	3	monkeys	mos-quitos	Africa, Americas, Asia, Carib-bean, Pacific	6-30%	none	supportive	(Basler, 2019; Flint *et al.*, 2014; Grard *et al.*, 2007; Iannetta *et al.*, 2019; Marty *et al.*, 2006; Pattnaik, 2006; Ruzek *et al.*, 2010; Shah *et al.*, 2018)
Yellow fever virus (YFV)	Yellow fever	3	monkeys	mos-quitos	Africa, South Americas	First phase: 10-20% Toxic phase: 50-70%	licensed	supportive	
Kyasanur Forest fever virus	Kyasanur Forest fever	3	rodents	ticks	South India	with HF: 50%	none	supportive	
Omsk HF (OHF) virus	OHF	3	rodents	ticks	West/ North Siberia, Kazakh-stan	1-3%	none	supportive	

What all these viruses (Table 1) have in common is that they are enveloped (lipid bilayer) viruses containing a single-stranded ribonucleic acid (RNA) as the genome. The morphology displays various forms and sizes, ranging from small isometric shapes, to medium spherical virions, but can also emerge in atypical filamented or pleomorphic forms (Marty *et al.*, 2006). All VHF viruses are classified as BSL-3 or BSL-4 agents and are able to cause life threatening diseases with high mortality rates. Zoonotic agents can be spread by aerosols (except for Dengue virus (DENV)) and, thus, transmission is not only possible via the fecal-oral route, but also via the respiratory tract (Marty *et al.*, 2006; Racsa *et al.*, 2016). Transmission from hosts to

humans occurs through direct contact with body fluids from infected hosts such as rodents, ticks, livestock, bats or monkeys, or by vectors such as mosquitos and ticks (Cleri *et al.*, 2007; Marty *et al.*, 2006). Person-to-person infections are possible for most of the VHF agents, such as EBOV, MARV, LASV and Crimean Congo haemorrhagic fever virus (CCHFV). In hospitals, patients and staff can easily become infected through contact with infected blood and body fluids or by the inhalation of contaminated aerosols (Iannetta *et al.*, 2019; Marty *et al.*, 2006; Zakham *et al.*, 2019).
All patients infected with an agent causing VHF initially show non-specific symptoms, such as high fever (>38.5°C) and general discomfort. Molecular methods are the gold standard for laboratory confirmation, but due to the short viremia phase, RNA can only be detected for several days. The detection of Immunoglobulin (Ig) M in human samples by using serological methods is mandatory for the diagnosis of acute VHF. IgM is the first humoral specific immune response after contact of the human immune system with the infectious agent (Racsa *et al.*, 2016).
The therapeutic options for patients suffering from an infection with VHF focus on symptomatic treatment. In case of mild forms, a doctor will conduct symptomatic treatment, whereas severe cases may require supportive treatment at the intensive care unit (*e.g.* maintenance of hydration, blood pressure, and application of supplemental oxygen) (Marty *et al.*, 2006). Unfortunately, there are currently no licensed antiviral drugs for the treatment of VHFs, but off-label use drugs are available. One of these is ribavirin, a non-immunosuppressive nucleoside analogue with a variety of antiviral properties (Sidwell *et al.*, 1979) and activity against several haemorrhagic fever viruses such as LASV (McCormick *et al.*, 1986), CCHFV (Johnson *et al.*, 2018), RVFV (Huggins, 1989) and haemorrhagic fever with renal syndrome (HFRS) induced by members of genus *Orthohantavirus* (Huggins, 1989). Ribavirin is not effective against *Filoviruses* (EBOV and MARV) and *Flaviviruses* (DENV, YFV, OHFV and Kyanasur Forest disease virus) (Marty *et al.*, 2006). There is an approved vaccine against YFV (Monath, 2005) and several candidates against EBOV, MARV (Suschak and Schmaljohn, 2019) and LASV (Fischer *et al.*, 2021) are in preparation. Two vaccines are available against Argentinian haemorrhagic fever (HF) (McKee *et al.*, 1992), Bolivian HF (Maiztegui *et al.*, 1998) and Rift Valley fever (Rusnak *et al.*, 2011), and vaccines for humans against *Orthohantavirus* are commercially available in China and Korea (Falzarano and Feldmann, 2013; Schmaljohn, 2012). In the United States of America (USA), a vaccine against Sin Nombre virus is used for rodents in endemic

areas to reduce the virulence in the hosts (deer mice, *Peromyscus maniculatuts*) by oral intake of the vaccine (Warner *et al.*, 2020).

4.3 Public health in Germany versus public health in emerging countries

When treating patients with FUO, their travel history and recent activities as well as their medical and dietary plan should be considered (Cleri *et al.*, 2007; Durack and Street, 1991; Wright and Auwaerter, 2020). A correct classification of FUOs should assist the physician in finding a potential cause for the FUO symptoms. In well and highly developed countries like Germany, FUO is not really a problem, since contemporary diagnostic methods are available that allow for a differentiated diagnosis. Furthermore, there is a network of reference or conciliar laboratories and commercial laboratories, which specialize in specific pathogens. With the state-run Bernhardt-Nocht-Institute in Hamburg, which specializes in human diseases, and the Friedrich-Loeffler Institute, which works on animal health diagnostics, the epidemiological surveillance of zoonotic agents is on a high level in Germany. This surveillance of zoonotic agents is further intensified, since many infections have to be reported to national databases according to the national infection protection act (Infektionsschutzgesetz IfSG, §6 Meldepflichtige Krankheiten) (Bundesministerium der Justiz and Bundesamt für Justiz, 2002). The general monitoring and surveillance of infections in Germany is carried out by the Robert-Koch-Institute based in Berlin.

In emerging countries such as the Central Asian Republic of Kazakhstan, all infections and parasitic diseases diagnosed by doctors are reported monthly to the Sanitary Practical Center for Sanitary-Epidemiological Expertise and Monitoring (SPC SEEM), which are then reported to the Ministry of Health (MoH). These monthly reports to the authorities include infections such as Anthrax, Tularaemia, Brucellosis, Leptospirosis, Crimean Congo haemorrhagic fever (CCHF), Tick-borne encephalitis (TBE), Lyme disease, Rickettsiosis, Plague, Leishmaniosis, Rabies, and HFRS. Some regions of Kazakhstan are declared as endemic regions for specific infectious diseases. Unfortunately, it is only in these endemic areas that doctors are aware of certain diseases and that reliable diagnostics for the identification of the infectious agents are available. In non-official-endemic regions, there are no reliable diagnostic methods. Hence, infections with these pathogens may not be detected and recorded.

4.4 Fever of unknown origin in Kazakhstan

Especially in emerging countries, FUO is a significant public health problem, due to the lack of appropriate diagnostic methods and inefficient treatment (Abdiyeva *et al.*, 2019). In these countries, there is a lack of suitable diagnostic tools, well-educated physicians and trained laboratory staff.

Kazakhstan is one of the countries and it is a country located in Central Asia. It borders China to the east, Turkmenistan to the south and Uzbekistan, Kyrgyzstan, Russia as well as the Caspian Sea to the west. Kazakhstan has a continental climate with a diverse landscape including forest-steppes, steppes, semi-deserts, deserts and mountain ranges (Atshabar *et al.*, 2010). The country itself is divided into 14 oblasts (=regions) with three major cities. It has approximately 19 million inhabitants, with a theoretical population density of seven inhabitants per km^2 - most people, however, are urban citizens ("Worldometer -Population Kazakhstan," 2022).

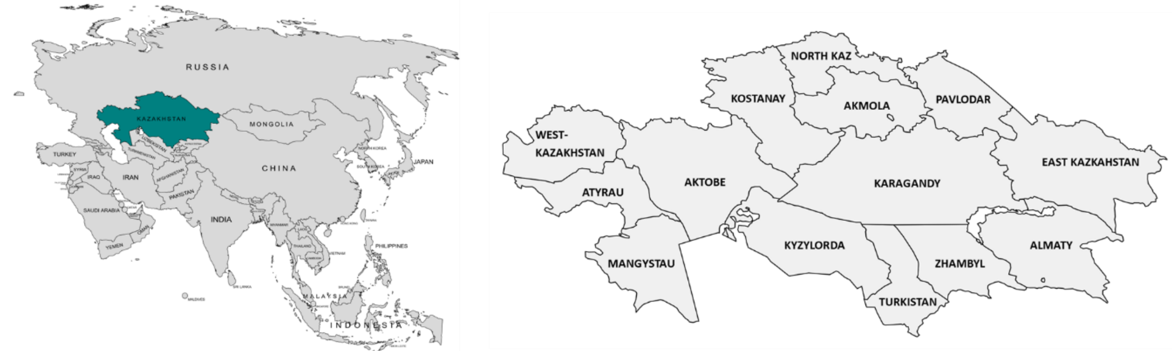

Figure 1: Kazakhstan (green) is a landlocked country located in Central Asia and is divided in 14 regions (=oblasts). Maps generated with mapchart.net ("mapchart.net," 2022).

Kazakhstan hosts several (unknown) natural foci of important zoonotic agents like, B. anthracis, CCHFV, F. tularensis, Leptospira, *Orthohantaviruses*, *Rickettsia* spp., TBEV and Y. pestis (Atshabar *et al.*, 2010). For this reason, 18 so-called anti-plague stations (APS) were established in different regions of Kazakhstan between 1914 and 1949. At the beginning, the main responsibility of these APS was the epidemiological and epizootic surveillance of plague and Y. pestis infection rates in their natural hosts and vectors (fleas of the genus *Xenopsylla*) (Melikishvili, 2006). Later, the APS evolved into regional observation centres for other particularly dangerous pathogens that are endemic to Kazakhstan, such as TBEV, CCHF, members of genus *Rickettsia* and *Orthohantaviruses* (Ben Ouagrham-Gormley *et al.*, 2006).

Because of the EDPs, pathogens are monitored by the APS throughout Kazakhstan and human cases of infection with TBEV, CCHFV, *Rickettsia* and other zoonotic

diseases are recorded by the “Scientific and Practical Center of Sanitary and Epidemiological Expertise and Monitoring” (SPC SEEM). The distribution of these EDP agents in vectors, hosts and humans is still poorly explored. As mentioned above, only in endemic areas are physicians aware of the diseases and may recognize them based on the clinical picture. Diagnostics mainly consist of clinical anamneses, due to the lack of reliable diagnostic methods in Kazakhstan. Especially in patients suffering from mild forms of diseases with non-specific symptoms, the agents cannot be detected and these patients are diagnosed with FUO (Abdiyeva *et al.*, 2019; Peintner *et al.*, 2021; Shin *et al.*, 2022; Tukhanova *et al.*, 2019; Turebekov *et al.*, 2021).
This present work will focus on several viral zoonotic agents such as members of the *Flaviviridae*, like OHFV, TBEV and WNV, but also members of the genus *Orthohantavirus*. All of them have an impact on the health care system in Kazakhstan.

4.5 Family of *Flaviviridae*

The family of *Flaviviridae* consists of four genera: *Flavivirus* (53 species), *Hepacivirus* (14 species), *Pegivirus* (11 species) and *Pestivirus* (11 species) (ICTV, 2020). This family is characterized by enveloped virions of 40-60 nm in size with positive-sense, non-segmented RNA genomes ranging from 9.0-13 kilobases. The genome holds a single open reading frame (ORF) flanked by 5’- and 3’-terminal untranslated regions (UTR), forming specific secondary structures that are necessary for replication and translation of the genome. For the genus *Flavivirus*, the initial translation is cap dependent. For the remaining genera *(Hepacivirus, Pegivirus* and *Pestivirus*), internal ribosome entry site elements are responsible. The genomic RNA encodes three structural proteins (envelope (E), precursor (pr) membrane (M) and capsid (C)), and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Replication and synthesizing of the polyprotein happen in the cytoplasm of host cells. The polyprotein is subsequently co- and post-translationally cleaved by viral- and host-dependent proteases to generate the operational individual proteins. (Mukhopadhyay *et al.*, 2005; Neufeldt *et al.*, 2018; Simmonds *et al.*, 2017).
The biggest of the genera with 54 species is the genus of the *Flaviviruses*, which belongs to the group of arthropod-borne viruses as they are associated with mosquitos or ticks (Mukhopadhyay *et al.*, 2005). Primary hosts during the life cycle of ticks and mosquitos are vertebrates and birds and the symptoms of the infections range from asymptotic, mild to fatal with haemorrhagic fever or neurological infections. The main

representative agents affecting human beings are TBEV, WNV, YFV, DENV or Japanese encephalitis virus (JEV). In addition, other members of the genus can infect domestic and wild animals and may have an economical or environmental effect such as the Usutu virus which affected the Eurasian blackbird (*Turdus merula*) population (Giglia *et al.*, 2021).

4.5.1 Omsk haemorrhagic fever virus

Omsk haemorrhagic fever virus (OHFV) is the causative agent of Omsk haemorrhagic fever (OHF), which also causes VHF. OHFV belongs to the family of *Flaviviridae* and more specifically to the tick-borne encephalitis (TBE) complex of *Flaviviruses* (Im *et al.*, 2020).

For a long time, OHF was known only to be present in Kurgan, Tyumen, Omsk and the Novosibirsk region of Russia (Kovalev *et al.*, 2021; Ruzek *et al.*, 2013, 2010). In humans it shows a biphasic clinical course. In the beginning, non-specific symptoms similar to those of the common flu are followed by primary symptoms such as chill, cough, gastrointestinal symptoms, headache, muscle pain, nausea, fever (39-40°C) and haemorrhages. The bleeding can occur at the skin, but nasal, gingival and uterine bleeding, as well as subconjunctival haemorrhages (broken blood vessel in the eye) are observed, too. While 50-70% of patients recover after this first phase, others may experience a second, more severe phase of the disease with encephalitic symptoms, such as headache and meningitis, in addition to the primary symptoms (Akhrem-Akhremovich R.M., 1948; Ruzek *et al.*, 2010). OHF has a low case fatality rate (CFR) (0.5-3%) ("CDC," 2022) and very often goes undetected due to mild and subclinical courses (Belov *et al.*, 1995; Ruzek *et al.*, 2010).

Human-to-human transmission is not known to date. Additionally to tick bites (*Dermacentor (D.) reticulatus* and *D. marginatus*), infected rodents and small mammals are the transmitters of OHFV to humans (Chumakov, 1948; Gagarina, 1957; Gagarina and Netsky, 1955; Ruzek *et al.*, 2013). The muskrat *Ondatra (O.) zibethicus* is the main host of OHFV. The small mammal is not native in Russia and was imported from Canada for the production of fur in the late 1940ies (Ruzek *et al.*, 2010). By now, it is common and widespread throughout North America, Europe, the Balkans, Russia and Central Asia, including Kazakhstan ("GBIF," 2022).

The feces of OHFV infected *O. zibethicus* contain a large amount of OHFV-RNA. Other small mammals and rodents get infected by inhalation of contaminated aerosols or by

contact with/intake of water, which has been contaminated by the feces or even by the carcasses of infeceted *O.zibethicus*. OHFV is stable for more than 14 days in summer and for more than three months in winter (Kharitonova and Leonov, 1986; Ruzek *et al.*, 2013).

4.5.2 Tick-borne encephalitis virus

Tick-borne encephalitis virus (TBEV) is one of the most common tick-borne viral pathogens. Most of the TBEV infections show an asymptomatic subclinical course. Those with symptomatic courses, however, lead to tick-borne encephalitis (TBE) with neurological symptoms such as encephalitis, meningitis and meningoencephalitis. In severe cases, these can lead to lifelong neurological problems or even death. The case fatality of TBE is dependent on the TBE-subtype, namely the European (TBEV-Eu, CFR: 0.5-2%), the Siberian (TBEV-Sib, CFR:2-3%) and the Far-Eastern subtype (TBEV-FE, CFR: 40%) (Bogovic and Strle, 2015; Grard *et al.*, 2007; Heinze *et al.*, 2012; Taba *et al.*, 2017).

It is a spherical shaped lipid-enveloped RNA virus and composed as all members of the Flavivirus genus of three structural proteins E, M and C (Bogovic and Strle, 2015). The virus is transmitted by bites of so-called hard ticks (family *Ixodidae*, characterized by having a hard shield or scutum), such as *Ixodes (I.) ricinus* in Europe, *I. persulcatus* in Eastern Europe, Russia and far-east Asian countries (Mansfield *et al.*, 2009) and *I. ovatus* in Japan (Takeda *et al.*, 1998). Roughly one percent of all human infections with TBEV can be ascribed to the consumption of non-pasteurized milk or milk products from TBEV-infected livestock (Holzmann *et al.*, 2009; Hudopisk *et al.*, 2013). Pasteurization inactivates the protein E, which is the main antigen for inducing the production of neutralizing antibodies (Bogovic and Strle, 2015; Kaiser, 2008).

4.5.3 West Nile virus

West Nile virus (WNV) belongs to the genus *Flavivirus* and is, together with St. Louis encephalitis virus, Murray Valley encephalitis virus (MVEV) and JEV a member of the JEV serogroup (antigenic complex). All members of the JEV serogroup are human pathogens, except for WNV, MVEV and JEV, which are animal pathogens transmitted by mosquitos (Petersen *et al.*, 2013). WNV leads to West Nile fever, which can be found in regions of Asia, Africa, Americas and Europe (Lafri *et al.*, 2019). At least five lineage strains of WNV are known, but so far only two of them are pathogenic linages:

WNV-1 and WNV-2. WNV-2 is found in mosquitos in sub-Saharan African countries and Europe and infects mainly horses. WNV-1 is distributed in Europe and North America and leads to viral encephalitis in horses and also in humans (Petersen *et al.*, 2013).

During the natural life cycle wild birds and mosquitos (*Culex* spp.) are affected (Kramer *et al.*, 2008). WNV is particularly dangerous for birds of the family *Corvidae,* where the infection leads to a severe inflammation, followed by tissue necrosis, bleeding of internal organs and infection of the central nervous system (CNS). Horses are also often infected with WNV, but only with moderate injuries to the CNS (Petersen *et al.*, 2013).

Humans become infected through mosquitos, organ transplantation or blood transfusions. While most of the human infections are asymptomatic or show non-specific flu like symptoms, severe cases can result in meningitis or meningoencephalitis. To date, there is neither a vaccine nor a specific treatment for humans. Only supportive treatment can alleviate the symptoms of the patients (Colpitts *et al.*, 2012).

4.6 The genus *Orthohantavirus*

The genus *Orthohantavirus* is part of the order *Bunyavirales* and includes 38 species (ICTV, 2020). The spherical or oval virions are 80-120 nm in diameter and consist of a tripartite negative sense RNA genome. The large segment encodes viral RNA-dependent RNA polymerase (6.5 kb) that functions as transcriptase and replicase. The medium segment (3.6 kb) contains a viral glycoprotein precursor, which is cleaved into two glycoproteins called Gn and Gc. The small segment (1.6-2 kb) produces the viral nucleocapsid protein, which is associated as a multimer with the viral RNA genome (Schmaljohn and Hjelle, 1997).

Persistently infected rodents carry *Orthohantavirus* and spread it through their feces. Transmission to humans occurs through inhalation of virus-contaminated aerosols or, in rare cases, through direct contact between humans and infected rodents (Essbauer and Krautkrämer, 2015; Heyman *et al.*, 2011). In Eurasia, *Orthohantavirus* infections predominantly cause haemorrhagic fever with renal syndrome (HFRS). In Germany and Fennoscandia, the very mild form of HFRS is also known as *nephropathia epidemica* (NE). In contrast, in the Americas, *Orthohantavirus* causes Hantavirus

pulmonary syndrome (HPS), which is also called Hantavirus cardiopulmonary syndrome (HCPS) (Jonsson *et al.*, 2010).
HFRS can develop into mild, moderate or severe forms with a CFR of 5-15%. The severity of HFRS varies depending on the species of Hantaviruses. Basically, Hantaan orthohantavirus (HTNV) causes a severe form of HFRS (CFR: 15%). Dobrava-Belgrade orthohantavirus (DOBV, including various genotypes such as Dobrava, Saaremaa, Kurkino and Sochi) leads to a moderate to severe form (CFR: 5%) in Asian countries. The globally distributed Seoul orthohantavirus (SEOV) causes mild forms of HFRS, similar to Puumala orthohantavirus (PUUV), which is mainly endemic in Europe, where the resulting disease is also known as *nephropathia epidemica* (NE, CFR:<1%) (Avšič-Županc *et al.*, 2015; Jiang *et al.*, 2017; Jonsson *et al.*, 2010; Klempa *et al.*, 2013; Krautkrämer *et al.*, 2016; Lednicky, 2003; Rasche *et al.*, 2004; Vaheri *et al.*, 2013). Tula orthohantavirus (TULV) can also found in its hosts, such as *Microtus arvalis* all over Europe, the Baltic states and Western Russia. It causes HFRS only in an asymptotic or mild form and only few human cases of TULV are reported (Klempa *et al.*, 2003).

4.6.1 Clinical course of haemorrhagic fever with renal syndrome

Typically, HFRS is divided into five phases (febrile, hypotensive, oliguric, polyuric and convalescent), which can be differentiated clearly during severe clinical courses and may be absent in mild forms of HFRS and in NE (Jiang *et al.*, 2017; Lednicky, 2003; Schmaljohn and Hjelle, 1997). The typical clinical manifestation of HFRS starts with flu-like symptoms, hyperaemia of the face, neck and upper body, insomnia, followed by hypotension and haemorrhagic syndrome (petechial skin rash, bleeding, haemorrhages). Later, patients develop severe thrombocytopenia and an acute kidney injury. These symptoms are accompanied by leucocytosis, elevated creatinine, urea, haematuria and proteinuria (Vaheri *et al.*, 2013). Additionally, patients may develop organ manifestations such as lumbar pain, costovertebral angle tenderness (Murphy’s punch signs), vomiting, abdominal pain, face puffiness, eyelid swelling and hyperazotemia (increased level of nitrogen-containing compounds such as urea and creatinine) (Park *et al.*, 2011). However, a diversification of potential symptoms of *Orthohantavirus* infections can be observed in the more recent literature. The canonical clinical signs of Eurasian representatives of *Orthohantavirus* causing HFRS have also changed to pulmonary forms (Huang *et al.*, 2020; Rasmuson *et al.*, 2011).

Typically, patients with HFRS receive supportive fluids, electrolytes, symptomatic therapy and short-term cortisol therapy in order to reduce shock. Another treatment option is to support kidney function, blood pressure and oxygen supply (Jiang *et al.*, 2017).

4.7 Bacterial zoonotic infections by *Rickettsia* species

Zoonotic diseases are not only transmitted by viruses but also by bacterial agents. Part of this work included a study of the distribution of bacteria of the genus *Rickettsia.* These intracellular parasites live in the cytosol of their hosts and are small gram-negative bacteria measuring 0.3 to 0.5 by 0.8 to 2.0 µm (Blanton, 2019; William B. Whitman, 2009). Their genome is rather small at 1.1-1.5 megabases. With regard to their species, *Rickettsia* are categorised by multilocus sequencing typing (MLST). With over 1200 validated *Rickettsia* and numerous non-validated species, the *Rickettsiaceae* family is one of the most diverse bacterial families (Schoch *et al.*, 2020). Validated *Rickettsia* species can be divided into four species groups (Table 2): 1) spotted fever group (SFG), 2) typhus group (TG), 3) transitional group and 4) ancestral group.

Table 2 Overview of some *Rickettsia* species and their diseases transmitted by their vectors and their distribution.

Disease	Organism	Distribution	Vector	References
Spotted Fever Group (SFG)				
Rocky Mountain spotted fever	*R. rickettsii*	the America	tick (*Dermacentor* spp.; *Rhipicephalus sanguineus, Ambylomma* spp.)	(Blanton, 2019)
Mediterranean spotted fever	*R. conorrii*	Europa, Africa, Asia	tick (*R. sanguineus*)	(Blanton, 2019; Colomba *et al.*, 2006)
Siberian tick typhus	*R. sibirica*	Eurasia, Africa	tick *(D. nuttalli, D. marginatus, Haemaphysalis concinna*)	(Parola *et al.*, 2005)
Queensland tick typhus	*R. australis*	Australia	tick *(Ixodes* spp.*)*	
unnamed	*R. helvetica*	Europe, Asia	tick *(Ixodes* spp.*)*	

Typhus Group (TG)				
Epidemic Typhus	*R. prowazekii*	South America, Africa, Eurasia	louse *(Pediculus humanus corporis*)	(Bishop-Lilly *et al.*, 2013; Blanton, 2019)
Murine typhus	*R. typhi*	worldwide	flea (*Xenopyslla cheopis)*	(Blanton, 2019; Eremeeva *et al.*, 2008)
Transitional Group				
Rickettsialpox	*R. akari*	North America, Eurasia	mite (*Liponyssoides sanguineus)*	(Blanton, 2019)
Flea borne spotted fever	*R. felis*	worldwide	flea (*Ctenocephalides felis)*	
Ancestral Group				
Non pathogenic	*R. bellii*	the Americas, China	Mosquitos (*Culex* spp.*, Aedes, Anopheles*), Ticks (*Dermacentor* spp., *Haemaphysalis* spp.*, Ornithodoros* spp. *Argas* spp. *Ixodes* spp. *Amblyomma)*	(Li *et al.*, 2022; Parola *et al.*, 2013)
Non pathogenic	*R. canadensis*	North-, Central America	*Haemaphysalis leporispalustris*	(Parola *et al.*, 2013)

The first species group is the tick-borne spotted fever group (SFG), which includes more than 30 species. At least 15 of them lead to diseases in humans, such as the Rocky Mountain spotted fever (RMSF, *R. rickettsia*), the Mediterranean spotted fever (*R. conorrii*), Siberian tick typhus (*R. sibirica*) and Queensland tick typhus (*R. australis*) (Blanton, 2019; Brown and Macaluso, 2016; Colomba *et al.*, 2006; Graves and Stenos, 2009; Nanayakkara *et al.*, 2013; Niang *et al.*, 1998; Parola, 2004; Robinson *et al.*, 2019). The second major group is the typhus group (TG). Its agents are associated with lice and fleas, which cause epidemic typhus (*R. prowazekii*) and murine typhus *(R. typhi)* in humans (Bishop-Lilly *et al.*, 2013; Blanton, 2019; Eremeeva *et al.*, 2008; Newton *et al.*, 2019; Robinson *et al.*, 2019; Vallee *et al.*, 2010). The third species group is the transitional group. Infections in humans are caused by *R. akari* causing Rickettsialpox and *R. felis* causing flea-borne-spotted fever) (Blanton, 2019). The ancestral group, to which *R. bellii* and *R. canadensis* belong as members, is the fourth

Rickettsia group. It is the only group that does not have the ability to cause disease in humans (Li *et al.*, 2022; Parola *et al.*, 2013).
Species of *Rickettsia* are transmitted by bloodsucking arthropods such as fleas, ticks, mites and mosquitos. SFG species are transmitted by the bite of an infected tick (mainly hard ticks like *Ixodidae (Ixodes* spp., *Dermacentor* spp*., Rhipicephalus* spp*.)* and some soft ticks (*Argasdidae*). Characteristic clinical manifestations of SFG rickettsioses include fever, inoculation eschars and a generalized dermal rash. In addition, other non-specific flu-like symptoms such as cough, febrile temperatures, myalgia, widespread lymphadenopathy, abdominal ache and CNS infections are possible. In contrast, epidemic typhus and murine typhus of the typhus group (TG) cause high fever, headache and rashes on trunk and extremities combined with nonspecific symptoms like cough, myalgia and malaise. Additional neurological manifestations, like headache, meningitis and encephalitis, are also reported. Rickettsioses can cause CNS infections in humans, ranging from meningitis to fatal encephalomyelitis (Parola *et al.*, 2013; Raoult *et al.*, 2004; Sekeyová *et al.*, 2019).

Rickettsioses occur worldwide (Blanton, 2019). There are sparse reports of the disease and the prevalence of tick-borne infections such as rickettsioses in Asian countries, but it is known that SFG and TG *Rickettsia* are present in south-east Asia (Aung *et al.*, 2014; Parola *et al.*, 2013; Robinson *et al.*, 2019, 2018; Rodkvamtook *et al.*, 2018).

4.8 The human response to a viral or bacterial infection and how to detect it

Viruses and bacteria have developed several strategies to infect their hosts. Upon attachment of viruses at cellular surfaces, viruses can enter into host-cells by various endocytic mechanisms, such as chlathrin-mediated endocytosis, macropinocytosis or caveolar/lipid-raft-mediated endocytosis, but also by pore formation (Canton, 2018; Cossart and Helenius, 2014). Cellular signalling mechanisms are already induced during the initial contact of the virus with the host cell, which on the one hand trigger an antiviral immune response, but also support virus uptake, replication and spread. Regardless of the mechanism of internalisation viral genome replication starts in the cytoplasm or nucleolus followed by the assembly and release of a new virus on the host cell surface (Cossart and Helenius, 2014).
Similarly, bacteria adhere to host cell receptors or to extracellular matrix ligands. This results in the activation of host cell signalling pathways regulating antibacterial defence, but also bacterial uptake, in some cases (Barocchi *et al.*, 2005). The phase

in which viral (viremia) or bacterial (bacteremia) genome material is present in the host cell is rather short at three to four days. Only in this period of time molecular biological methods like PCR are able to detect the viral RNA or bacterial DNA (Figure 2) (Drosten *et al.*, 2003; Yamamoto, 2002).

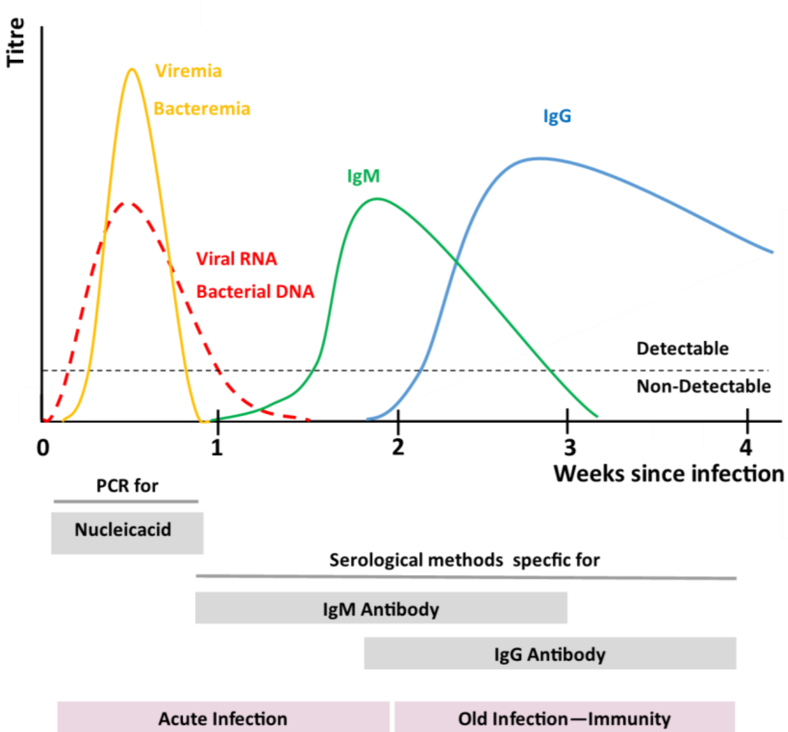

Figure 2 Antibody response after infection with a viral or bacterial agent. After an infection, viral RNA and bacterial DNA (red dotted line) are detectable by molecular methods like polymerase-chain-reaction (PCR) during the usually rather short viremia/bacteremia-phase (yellow line). After an infection is detected, the adaptive immune system responds and specific immunoglobulin (Ig) M antibodies (green line) are produced, followed by IgG antibodies (blue line). Antibodies can be detected by serological methods such as a pathogen specific IgM and IgG enzyme-linked immunosorbent assay (ELISA). Detection of viral RNA/bacterial deoxyribonucleic acid (DNA) and IgM antibodies indicates an acute infection, whereas a detection of IgG antibodies indicates an old infection and an already achieved immunity against a specific pathogen.

After the entry of the viral or bacterial pathogen into the host, the innate and subsequently the adaptive immune system are activated. In contrast to innate immunity, which has a low specificity and is carried in particular by granulocytes and macrophages, adapted immunity produces specific antibodies against the pathogen. The machinery of the adaptive immune system is activated after the pathogen (virus or bacteria) has entered the host and is recognized and phagocytosed by dendritic cells and macrophages. In this step, the pathogen is enzymatically lysed in and subsequently fragments of the antigen are presented by receptors on their surface. Corresponding T helper cells bind to the presented antigens and get activated. This leads to two main reactions: 1) activation of the cytotoxic T cells to lyse infected cells

or 2) activation of B cells to generate the humoral immune response to produce and secrete immunoglobulin (Ig) M (Figure 2). When detected by IgM-specific ELISA, they indicate an acute infection with a pathogen, especially in combination with detection of viral RNA/bacterial DNA by PCR. After a few days or weeks, the immune response maturates and highly specific IgG antibodies are secreted. The detection of IgG indicates not only a later stage of an ongoing infection, but also an earlier encounter with the pathogen (previous infection or vaccination) (Figure 2) (Charles A Janeway *et al.*, 2001).

The gold standard for the detection of viral or bacterial infections in patients is either direct detection of the pathogen (by PCR – conventional or real-time) or indirect by screening with pathogen specific serological techniques (Racsa *et al.*, 2016).

ELISA is a sensitive and specific serological tool to detect molecules of interest. In short, a microtiter plate is enriched with virus-specific antigens. The patient sample (blood, urine) is added and if the sample contains IgG or IgM antibodies, they bind specifically to the immobilized antigens. Then, a second antibody is added, which binds to the first antibody. The second antibody is coupled to an enzyme (*e.g.*, horseradish peroxidase), which makes the complex visible via a colour reaction. In the final step, a substrate is added, which is converted into a dye by the enzyme. The stronger the colour, the more antibodies are in the patient's sample (Engvall and Perlmann, 1971).

Like the ELISA, the indirect immunofluorescence assay (IIFA) is also based on an antibody-antigen reaction. Various antigens are present on a solid plate. The antibodies to be detected from patient samples first bind to antigens fixed to a solid phase. A primary antibody is added to the sample, which binds specifically (lock-and-key principle) to the antigen in the sample. A second antibody combined with a fluorescent molecule is added to the probe, which binds specifically to the primary antibody. Therefore, the molecules of interest are combined with a fluorescent dye molecule and can be determined with an immunofluorescence microscope using light at a dye-specific wavelength. Employing IIFA enables the detection of a broad spectrum of antibodies. It is a useful tool to screen human serum samples for potential infectious diseases, but require experience by the user to evaluate them correctly. In contrast the ELISA is more user-friendly and also easier to evaluate than IIFAs.

However, serological diagnostics with ELISA and indirect IIFA have several shortcomings in terms of specificity for identifying antibodies reactive for specific viruses. This is caused by a cross-reactivity between closely related viruses, especially

within *Bunyaviridae* and *Flaviridae*. In addition, confirmation of serological results by a reference laboratory is recommended (Iannetta *et al.*, 2019; Racsa *et al.*, 2016). Instead of separate PCR reactions, multiplex assays have been available since 2002 that allow simultaneous detection of CCHFV, DENV, EBOV, LASV, MARV, RVFV, and YFV in one PCR (Drosten *et al.*, 2002). Furthermore, microarray-assays have been developed for broad-range detection of up to 84 pathogens by spotting a solid matrix is spotted with oligonucleotides representing specific genes of different pathogens. Samples of interest are marked with fluorescence molecules and after annealing, the intensity of the positive signal is compared with known positive signals (Leski *et al.*, 2009).

Lateral flow immunochromatographic assays for detecting antigens of *Orthohantavirus*, EBOV and DENV are commercially available. The test principle is comparable to the ELISA methods, but differs in that the immunological reaction is carried out on a chromatographic paper by capillarity. Two types of specific antibodies are used against the antigen of interest. The first one of the antibodies is immobilized on the chromatographic tissue, whereas the other antibody is labeled with colloidal gold. The mixture of the patient sample and the labeled antibody is loaded on the sample pad of the chromatographic paper. The antigen in the sample forms an immune complex with the antibody that is labeled with colloidal gold. The complex migrates along the chromatographic paper and comes into contact with the antibody immobilized on the membrane, forming a complex that results in a coloured line on the membrane indicating the presence of the antigen of interest in the patient's sample. Lateral flow assays are simple test devices designed to quickly and inexpensively detect the presence (or absence) of a molecule of interest in a sample (Koczula and Gallotta, 2016).

5 Aim of the work

Kazakhstan is the ninth biggest country in the world. Its huge areas of steppe and the hot and dry climate serve as a perfect cradle for many dangerous pathogens like *Y. pestis* (causing plague), *F. tularensis* (tularaemia), *B. anthracis* (anthrax), and many more. Epidemiological surveillance is performed in regional laboratories. However, they operate in regards of biosecurity and biosafety on levels from the former Soviet Union. Hence, safe laboratories, as well as proper and reliable modern diagnostics methods are missing. This leads to a lack of information about the spread of many zoonotic diseases and its agents such as members of the *Flaviviridae*-family, members of the genus *Orthohantavirus* but also of bacterial zoonotic agents such as *Rickettsia* species in patients, host (*e.g.,* small mammals) and vectors (ticks).
Patients with symptoms of an infection are usually only diagnosed by the clinical picture and no laboratory confirmation is available. When patients show unclear symptoms the aetiological agent often remains unknown. Kazakhstan is aware of this situation but is dependent on third-part funding by other countries to build up these capacities.

One aim of this work was to estimate the prevalence of several zoonoses, such as Omsk haemorrhagic fever virus and West Nile virus in human patients with fever or meningitis of unknown origin.

A second aim of this work was to detect several agents of zoonoses like *Rickettsia* species and *Orthohantavirus* species in their hosts.

The third aim of this work was to assess the minimum infection rate of potential vectors such as ticks and hosts such as small mammals for Omsk haemorrhagic fever virus.

Several methods, for example preparation of specimens for nucleic extraction, nucleic extraction, serological testing of human samples by ELISA and IIFT, real-time PCRs and conventional PCRs for detecting viral RNA or genomic and bacterial DNA and preparation of samples for sequencing had to be implemented in several laboratories in Kazakhstan. Devices, supply material, personal protective equipment and analytical kits had to be transported from Germany to Kazakhstan. Samples were commercially sequenced, and sequences were evaluated in Germany. Ethical approval for human and rodent studies was given by national authorities in Almaty, Kazakhstan and the Ludwig Maximilian University, Munich, Germany.

6 Published original papers

6.1 First Indications of Omsk haemorrhagic fever virus beyond Russia

Edith Wagner, Anna Shin, Nur Tukhanova, Nurkeldi Turebekov, Talgat Nurmakanov, Vitaliy Sutyagin, Almas Berdibekov, Nurbek Maikanov, Ilmars Lezdinsh, Zhanna Shapiyeva, Alexander Shevtsov, Klaus Freimüller, Lukas Peintner, Christina Ehrhardt and Sandra Essbauer.

Viruses, 14, 754-766, 2022

Article

First Indications of Omsk Haemorrhagic Fever Virus beyond Russia

Edith Wagner [1,2], Anna Shin [3,4], Nur Tukhanova [3,4], Nurkeldi Turebekov [4], Talgat Nurmakhanov [4], Vitaliy Sutyagin [5], Almas Berdibekov [5], Nurbek Maikanov [6], Ilmars Lezdinsh [5], Zhanna Shapiyeva [7], Alexander Shevtsov [8], Klaus Freimüller [2], Lukas Peintner [2,*], Christina Ehrhardt [1] and Sandra Essbauer [2]

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Abstract: *Omsk haemorrhagic fever virus* (OHFV) is the agent leading to Omsk haemorrhagic fever (OHF), a viral disease currently only known in Western Siberia in Russia. The symptoms include fever, headache, nausea, muscle pain, cough and haemorrhages. The transmission cycle of OHFV is complex. Tick bites or contact with infected small mammals are the main source of infection. The Republic of Kazakhstan is adjacent to the endemic areas of OHFV in Russia and febrile diseases with haemorrhages occur throughout the country—often with unclear aetiology. In this study, we examined human cerebrospinal fluid samples of patients with suspected meningitis or meningoencephalitis with unknown origins for the presence of OHFV RNA. Further, reservoir hosts such as rodents and ticks from four Kazakhstan regions were screened for OHFV RNA to clarify if this virus could be the causative agent for many undiagnosed cases of febrile diseases in humans in Kazakhstan. Out of 130 cerebrospinal fluid samples, two patients (1.53%) originating from Almaty city were positive for OHFV RNA. Screening of tick samples revealed positive pools from different areas in the Akmola region. Of the caught rodents, 1.1% out of 621 were positive for OHFV at four trapping areas from the West Kazakhstan region. In this paper, we present a broad investigation of the spread of OHFV in Kazakhstan in human cerebrospinal fluid samples, rodents and ticks. Our study shows for the first time that OHFV can not only be found in the area of Western Siberia in Russia, but can also be detected up to 1.600 km away in the Almaty region in patients and natural foci.

Keywords: Omsk haemorrhagic fever; Republic of Kazakhstan; FUO; ticks; rodents; CSF; flavivirus; tick-borne encephalitis complex

1. Introduction

Omsk haemorrhagic fever (OHF) is a zoonotic infectious disease presently only reported in regions of the Russian oblasts (=regions) Kurgan, Tyumen, Omsk and Novosibirsk

in Siberia [1–3]. The infection is caused by *Omsk haemorrhagic fever virus* (OHFV), a positive-sensed, single-stranded RNA virus. The virus may infect humans as a dead-end host and can lead to a biphasic course [2]. During the incubation period, infected humans develop unspecific flu-like symptoms followed by the onset of the primary symptoms. These include headache, cough, nausea, chill and muscle pain, but also gastrointestinal symptoms, subconjunctival haemorrhage, nasal, gingival and uterine bleeding, as well as skin haemorrhages accompanied by fever (39–40 °C). Furthermore, the disease leads to extreme sensitivity of the skin (hyperaesthesia) and a petechial rash of the upper body [3,4]. A second phase occurs in 30–50% of patients and leads to encephalitic symptoms such as headache and meningitis, additionally to the primary symptoms. [3]. Patients may suffer from long-term consequences such as weakness, hearing loss, hair loss and mental health problems, in combination with the loss of neurological functions in rare cases [2,5]. The case fatality rate (CFR) of OHF is low (0.5% to 3%) [6], but due to mild cases, with non-febrile patients and only unspecific symptoms without any haemorrhages [3,7], many infections remain undiagnosed.

The first epidemic outbreak occurred in Russia between 1945 and 1958, with 972 confirmed cases and over 1500 suspected cases of OHF. Until 1998, more than 300 OHF cases were confirmed in the regions Tyumen, Kurgan, Omsk and Novosibirsk. Between 1970 and 1975, a study with 577 patients with fever of unknown origin (FUO) showed exposure to OHFV in 18%. The results showed that persons with the mean age of 20–40 years were mainly affected, regardless of sex, as well as 30% of children up to 15 years. Muskrat trappers, poachers and their family members are one of the major risk groups [3,6,8–11]. No further data about recent outbreaks of OHF in Russia or elsewhere are reported in journals, probably due to non-detected and mild courses [12].

OHFV belongs to the tick-borne encephalitis (TBE) complex of flaviviruses in the family of *Flaviviridae* [13]. Other members of the TBE complex are the *Alkhurma haemorrhagic fever virus, Gadgets Gully virus, Kyasanur Forest disease virus* (KDFV), *Tick-borne encephalitis virus* (TBEV), *Langat virus, Louping ill virus* (LIV), *Powassan virus* (POWV) and the *Royal Farm virus* [3,14–17]. The high sequence similarity of this group is displayed in the similar morphology, structure and replication mode of the virions. Despite their similar morphology and replication strategy, they cause a variety of clinical symptoms. LIV, POWV and TBEV cause encephalitis, whereas AHFV, KFDV and OHFV mainly cause haemorrhagic fever in humans [13]. Interestingly, OHFV is one of the few viruses that can develop a clinical picture of combined haemorrhages and meningitis and, hence, represents a combination of both groups [2,8,13]. In detail, the 40-nm-large OHFV virion has a spherical or polygonal form, including a nucleolus of 25 nm. The nucleocapsid holds a positive-sense, single-stranded RNA genome wrapped in a capsid protein and is completely enclosed by a host-cell-derived lipid bilayer [11,18,19]. OHFV harbours a genome of 10,787 nucleotides (nt) with an open reading frame (ORF) of 10,242 nt [20]. The ORF is bordered by 5′ and 3′ untranslated regions (UTR).

The 5′ UTR has a length of 123 nt and contains a secondary structure in a conserved region of a stem loop and an additional stem loop at the 5′ end. However, this additional stem loop was shown to be 29 nt different from other representatives of the TBE complex [9,21]. The 413-nt-long 3′ UTR is shorter compared to other flaviviruses and includes a small variable region and a large core region that is highly conserved. Furthermore, a 3′-poly(A) tail is absent, similar to many other TBEV strains [9,21], and the 3′ UTR is conserved as in other flaviviruses of the TBE complex [9]. The ORF encodes a polyprotein of 3414 amino acids (aa) for three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). This polyprotein is cleaved at conserved cleaving sites during and after the translation process by cellular and viral proteases [9,11].

There are contrary opinions on the different subtypes of OHFV and the resulting phylogeny. Currently, there is a discussion on whether two or three subtypes can be distinguished. Early studies from the 1970s suggest that OHFV can be distributed into a minimum of three subtypes [22], which is backed by recent studies from 2015 [23] and

2021 [1]. However, others suggest that the OHFV reference strain *Bogoluvovska* (NC_005062) and the *Kubrin* strain are the same, due to a mix-up of samples in the laboratory [1]. Nevertheless, based on a recent finding, it can be assumed that OHFV circulating in vectors and hosts is very variable and differs markedly from the very early isolated strains [1].

No matter how diverse the OHFV pedigree actually is, the mode of transmission is similar to other members of the genus *Flaviviridae*. It is transmitted to humans by tick bite or via direct contact with infected animals, through respiratory or nutritive pathways. No person-to-person transmission has been reported so far [2,24]. The tick *Dermacentor reticulatus* (termed *D. pictus* in former USSR [25,26]) is the main host for OHFV in the forests and steppes of Siberia [2,24]. In the steppes of Southern and Western Siberia, OHFV is transmitted by *D. marginatus* [27,28]. Both *Dermacentor* ticks act as a parasite on several small mammals and birds in Russia. During their life cycle, they feed on small mammals, ungulates, domestic animals and humans. Risk groups that may be exposed to infected ticks are agricultural workers or individuals picking berries and mushrooms [3]. Further, the tick *Ixodes persulcatus* also plays a role in the life cycle of OHFV [2,29]. Additionally, OHFV was also isolated from mosquitos such as *Coquilletidia richiardii*, *Oligoryzomys flavescens* and *Ochlerotatus excrucians*, but so far, no data show their role in the ecology of OHFV [30].

OHFV can also be detected in and transmitted by mammals such as the muskrat (*Ondathra zibethicus*) [2]. The muskrat is common in North America, Europe, the Balkans, Russia and Central Asia, including Kazakhstan [31]. The excreta of infected muskrats hold a very high virus load of OHFV [2]. Natural foci of OHFV are typically steppes with lakes and marshes and forests. Many animals are infected by inhalation of the virus contained in aerosols and through contact with or consumption of water that is contaminated by the urine, faeces and/or dead bodies of infected muskrats. These wild hosts contract chronic infections, which sometimes are fatal. OHFV is stable in water for more than two weeks during summer and for more than three months in winter [2,29].

Currently, OHF infections and its agent OFHV are only reported and known in the Russian Federation [1,2]. Thus far, no studies on OHFV in other regions outside of Russia, especially in the neighbouring Republic of Kazakhstan, have been performed. Due to a progressing change in climate and, as a consequence thereof, the changing migration patterns of mammals and birds that may carry ectoparasites such as ticks, the virus can reach other areas than the currently known OHFV endemic areas.

The Republic of Kazakhstan is located in Central Asia and is adjacent to Russian Western Siberia. It borders China in the east, Turkmenistan in the south and Uzbekistan, Kyrgyzstan, Russia as well as the Caspian Sea in the west. The climate in Kazakhstan has a continental character. Kazakhstan itself is divided into 14 regions with three major cities. It has approximately 19 million inhabitants, with a theoretical population density of seven inhabitants per km^2—most people, however, are urban citizens [32].

In Kazakhstan, there are many cases of fever with unknown origin and many endemic viruses lead to diseases that have similar, non-specific symptoms [33–35]. In many cases, the agent cannot be detected because of the complexity and possible cross-reactions due to the close relationship of the flaviviruses within in the TBE complex [9,13,36].

We suggest that, due to the close geographic proximity to OHFV-endemic regions in Russia, OHFV might also be present in Kazakhstan. Hence, it should be possible to also detect OHFV in humans as well as reservoir hosts such as rodents and ticks in Kazakhstan. Here, we are conducting for the first time a broad investigation on the spread of OHFV in Kazakhstan by screening patient-derived cerebrospinal fluid samples (CFS) from Almaty, Akmola and East Kazakhstan and hosts such as ticks from North Kazakhstan, Akmola and Almaty and small rodents from the Almaty and West Kazakhstan regions. With these widespread areas, we are able to cover regions that are different in climate and in geographic and potential habitats, to gain more insight into the ecology and complexity of the transmission cycle of OHFV in Kazakhstan.

2. Materials and Methods

2.1. Human Sample Collection

In the years 2018 and 2019, serous meningitis patients with symptoms such as headache and/or meningeal signs were recruited for a cross-sectional study in the Republic of Kazakhstan in eight hospitals in the regions of East Kazakhstan, Akmola and in Almaty city [37]. After signing an informed consent form, patients' cerebrospinal fluid samples (CFS) were collected and taken on the day of hospitalisation and stored at −20 °C. This study was performed with the ethical approval of the ethics committees of the Kazakh National Medical University in Almaty, Kazakhstan (opinion number #565) and the Ludwig-Maximilians University in Munich, Germany (opinion number #19-373).

Each specimen was paired with a detailed, anonymised list of clinical symptoms such as meningitis, encephalitis, headache and/or nausea, vomiting and unconsciousness, as well as a questionnaire with questions regarding sociodemographic factors [37].

2.2. Tick Collection

Ticks were collected in three Regions of Kazakhstan (North Kazakhstan, Akmola and Almaty region) at eleven sampling regions, with, in total, 26 different sampling sites during the peak season of tick activity in May and June. In 2016 (for details, see [38]), 2018 and 2019, collections were conducted in the Akmola region in four sampling regions (Sandyktau, Zerendy, Ayrtau and Burabay regions) and in detail in five different villages and surroundings of the villages (villages of Sandyktau, Novonikolskoye, Sadovoye, Imantau and Katarkol). Collections at other sampling sites in the Almaty region, Almaty city and North Kazakhstan were performed in 2018 and 2019. Sampling regions in North Kazakhstan were located in five different areas (Ayirtau, Musrepov, Kyzylzharskiy and Zhumabayev regions, as well as in Petropavlovsk city) in nine different sites (villages of Priozernoye, Novoishymskoye, Anreyevka, Nezhenka, Konyuhovo, city of Petropavlovsk, regions Musrepov, Kyzykzharskiy and Zhumabayev).

The Almaty region was examined in five sampling areas (Alma-Arasan, along the Kapshagay highway, Butakovka, Kapshagay beach and Medeo) and Almaty city itself at seven sampling sites (Baumas Grove, Botanical Garden, grassland areas at the Bridge on Ryskulov and Suyunbay Street and in areas around four bus stations in Almaty city (Supplemental Table S1)).

All ticks were collected by flagging with white cotton sheets and stored in tubes at −20 °C corresponding to the sampling area. Later, ticks were morphologically identified following the official guidelines for tick identification in Kazakhstan [38–40]. After identification of the ticks (n_{ticks} = 4993 male and female adults), they were pooled in vials of 1–5 ticks (n_{pool} = 1058) and stored at −20 °C until further analysis.

2.3. Rodent Collection

In West Kazakhstan (19 trapping sites in the districts of Bayterek, Borili, Terekti and Taskala), in the Almaty region (city Tekeli, villages Rudnichniy and Bakanas) and in Almaty city, small rodents were trapped with snap traps and pork-fat-covered bread as bait during all seasons of 2018 and 2019 (Supplemental Table S2). Captured rodents were visually identified on the species level and, subsequently, the lung and brain tissue from 621 rodents were removed aseptically and stored at −20 °C in RNALater (Thermo Fisher Scientific, Waltham, MA, USA). Rodent trapping was conducted with the ethical approval of the Kazakhstan local ethics committee from the National Scientific Center for Especially Dangerous Infections in Almaty, Kazakhstan (protocol #4, 08.01.18) and the ethical committee of the Ludwig-Maximilians University in Munich, Germany (opinion number 18-631).

2.4. Homogenisation and Extraction of Viral RNA

Tick pools, rodent lungs and brain tissues were homogenised with two stainless steel beads and 1 mL of cell culture medium (GibcoTM MEM, Thermo Fisher Scientific,

Waltham, MA, USA) using the TissueLyser II (2 min at 30 Hz) (Qiagen, Hilden, Germany). The homogenised samples were centrifuged for 5 min at 2348× *g*. RNA was isolated from 140 µL of the supernatant using a commercial kit (QiAmp Viral RNA Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer's manual, and stored in aliquots at −80 °C [41].

2.5. Real-Time RT-PCR

Real-time reverse transcription (RT)-PCR was used to screen human liquor, tick, rodent lung and rodent brain tissue samples. Primers OHF-d1F (5′-GGCACARACCGTTGTTCTTG AGCT-′3) and OHF-d2R (5′-GCGTTCWGCATTGTTCCAWCCCACCAT-′3) and a TaqMan probe (5′-JOE-AGGTGTTCTGCTGTCTTGTCGAGCACCT-BQH1-′3) were used, detecting a conserved region within the envelope gene of OHFV RNA [2]. The primers bind the region from position 582 to position 720 and produce a fragment of 138 bp in length. The specificity of the used primers and the TaqMan probe was tested against OHFV strain *Bogolubovka* and other members of the TBE complex, such as *Langat virus*, *TBEV Far Eastern subtype Sofjin* and *LIV*. It was also tested against other flaviviruses, such as *Dengue virus (type 1–4)*, *Zika virus*, *Yellow-fever virus*, *West-Nile virus* (WNV), *Bagaza virus*, *Dakar bat virus*, *Kedougou virus*, *Spondweni virus* and *Usutu virus*, as well as other agents with the potential to cause Encephalitis, such as *Western Equine Encephalitis virus*, *Eastern Equine Encephalitis virus*, *Varicella Zoster virus* and *Herpes Simplex virus (type 1* and *2)* (Supplemental Table S3).

2.6. Statistical Analysis:

To calculate the prevalence of OHFV in the regions, the Minimum Infection Rate (MIR) method was applied, since the investigated flavivirus only shows a low prevalence (lower than 5%) in the investigated areas [42]. MIR is commonly used to estimate the proportion of infected individuals from pooled samples and is calculated as the ratio of the number of positive pools to the total number of ticks tested. It is assumed that only one infected tick exists in a positive tested pool. Since the arboviruses, which are transmitted via arthropods such as mosquitos and ticks, are relatively rare, the assumption is valid [43]. The MIR was calculated for each sampling site and individually for all tick species. Maps were generated using DIVA GIS [44] and www.mapchart.net (accessed on 16. January 2022).

3. Results

In this study, we aimed to identify OHFV, a member of the TBE complex virus family in CFS, in ticks and rodent tissue in the Republic of Kazakhstan. OHFV has a lot of sequence similarity to other closely related flaviviruses. To ensure the specificity of the published primers OHF-d1F and OHF-d2R, and the TaqMan probe targeting the E-gene of OHFV [2], which encodes the envelope protein, we tested them against isolates of several viruses and bacterial agents. The screening panel contained flaviviruses, especially members of the TBE complex, such as *Langat virus* and the *TBEV Far Eastern subtype strain Sofjnn*. Further, we screened for more remotely related flaviviruses including *Dengue virus (type 1–4)*, *Zika virus*, *Yellow-fever virus* and WNV (Supplemental Table S3). The primers did not bind to any of the tested flaviviruses, and also all other tested viruses and bacterial species were negative. Only the OHFV (strain *Bogolubovka*) specimen showed an expected strong signal. Hence, the used real-time RT-PCR assay is highly sensitive and can be exclusively be used for the detection of OHFV.

This establishment of a reliable diagnostic allows for the systematic screening of the existence of OHFV in Kazakhstan. As a proof of principle, we first concentrated on the analysis of a collection of human CFS specimens from patients with symptoms such as headache and meningism [37], being, hence, in the first episode of the typical OHF biphasic clinical course, where a high viral load can be expected.

Two out of the 130 CSF specimens tested positive for OHFV RNA (1.53%). Both patients were treated in the Almaty City Hospital (Figure 1). All 128 remaining CSF samples were negative for OHFV RNA.

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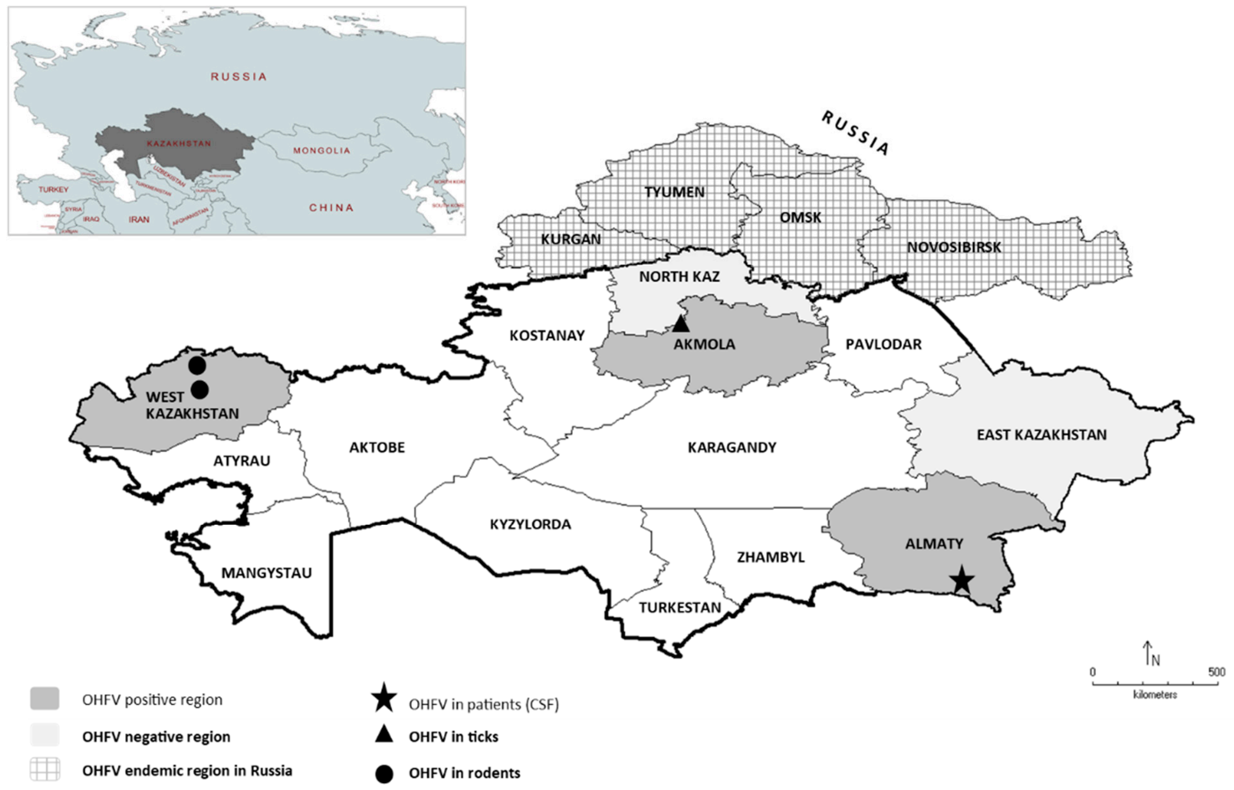

Figure 1. Investigation of Omsk haemorrhagic fever virus (OHFV) in Kazakhstan. Patients with suspected meningitis or meningoencephalitis in East Kazakhstan, Akmola and Almaty city, rodents from Almaty city, Almaty region and West Kazakhstan and ticks originating from North Kazakhstan, Akmola region, Almaty city and Almaty region were screened for OHFV RNA (light and dark grey). OHFV regions identified in this study (dark grey) with OHFV RNA-positive patients (★), ticks (▲) and rodents (●). In North Kazakhstan and East Kazakhstan, no OHFV was identified (light grey). Thus far, OHFV-endemic regions in Russia adjacent to Kazakhstan are Kurgan, Tyumen, Omsk and Novosibirsk (grey pattern).

The first patient was a 33-year-old male, born and living in Almaty city in a rental flat. He stated that he had no contact with any animals such as sheep, cattle, dogs or cats. Furthermore, he did not remember any tick bites. The clinical symptoms that he reported on the day of hospitalisation were headache and neck pain. His temperature, blood pressure and pulse rate were in a normal range. The second patient was a 22-year-old female, born in the Kyzylorda region, but now living in Almaty city in a city apartment. She had recently visited Kyzylorda but also did not remember any contact with animals or any tick bites. She was hospitalised with fever (>37.5 °C) and headache. The blood pressure was in normal boundaries but the pulse rate was increased (<80).

This detection of OHFV in Kazakhstan inhabitants prompted us to further screen for OHFV in their natural hosts such as ticks and rodents. In total, 4993 ticks from 26 sampling sites from three different regions (Akmola, North Kazakhstan and Almaty region), divided into 1058 pools, were investigated from three regions (Figure 1, light and dark grey). The investigated species were *Dermacentor marginatus* (n_{pools} = 575, n_{ticks} = 2762), *D. reticulatus* (n_{pools} = 456, n_{ticks} = 2167), *D. niveus* (n_{pools} = 6, n_{ticks} = 23) and *Ixodes persulcatus* (n_{pools} = 21, n_{ticks} = 41). In the Sandyktau area (Figure 1), screening of tick samples by using OHFV RT-PCR revealed one positive tick pool of *D. marginatus* from 2016 (1 positive pool out of 90 pools in total (1.1%), MIR of *D. marginatus* at village Sandyktau, Akmola region, in 2016: 0.01) and five positive pools of *D. reticulatus* originating from flagging in 2018 (5 out of 39 pools (12.8%), MIR of *D. reticulatus* at village Sandyktau, Akmola region, in 2018: 0.17). Furthermore, there were two OHFV RNA-positive pools from the Sadovoye area from 2018

(one pool of *D. reticulatus*, and one pool of *I. persulcatus* (2 out of 66 pools (3%), MIR of *D. marginatus* 0.02; MIR of *I. persulcatus* 0.5, both at village Sadovoye in Akmola, 2018). In total, of 21 investigated *I. persulcatus* tick pools, one in Akmola from the Sandyktau area at the village Sadovoye was positive for OHFV (1 out of 21 pools investigated in total, 4.76%) (Table 1). All positive pools originated from the Akmola region (Figure 1, dark grey areas, black triangle). All other tick pools from Akmola 2018 and 2019, North Kazakhstan 2018 and 2019 and the Almaty region 2019 were negative for OHFV-RNA (Figure 1, light grey areas).

Table 1. Results of OHFV real-time RT-PCR screen in tick pools of *D. marginatus, D. reticulatus*, *D. niveus* and *I. persulcatus* (in total, 1058 tick pools) from Almaty city (seven trapping sites), Almaty region (five trapping sites), Akmola region (six trapping sites) and North Kazakhstan (nine trapping sites).

Region	Year	Collected Species	Positive Species (#Of Pools)
Almaty region	2018/2019	1	0
Almaty city	2018/2019	2	0
Akmola region	2016/2018/2019	4	*D. marginatus* (2), *D. reticulatus* (6), *I. persulcatus* (1)
North Kazakhstan	2018/2019	4	0

The natural targets of ticks are small mammals. Therefore, 621 small rodents from two regions (Almaty and West Kazakhstan, Figure 1, light and dark grey areas) were included in this study in order to learn more about the natural reservoir of OHFV in Kazakhstan. Baiting with pork fat led to the capture of a broad range of rodents, including the family Muridae represented by *Apodemus uralensis* (*n* = 259), *Mus musculus* (*n* = 128) and *Rattus norvegicus* (*n* = 39), the family Gerbilinae represented by *Meriones meridianus* (*n* = 2), the family Cricetidae represented by *Microtus arvalis* (*n* = 86), *Microtus kirgisorum* (*n* = 49) and *Clethrionoyms glareolus* (*n* = 12) and the family Gliridae represented by *Dryomys nitedula* (*n* = 15). Additionally, insectivores such as *Crocidura suaveolens* (*n* = 28), Soricidae (*Sorex araneus* (*n* = 1), *Sorex minutus* (*n* = 1) and a not further classified *Sorex* spp. (*n* = 1)) were trapped and lung and brain tissue samples were screened for OHFV (Supplemental Table S2).

In three lungs of rodents of the species *C. glareolus*, captured in the Teretki District in West Kazakhstan in 2018, OHFV RNA was detected (*n* = 3/9, 33.3%) (Table 2). Furthermore, viral OHFV RNA was isolated from two lungs of *M. musculus* from the Taskala District (*n* = 2/16, 12.5%), in one specimen of *A. uralensis* from Oral City (*n* = 1/27, 3.7%), as well as from one specimen of *A. uralensis* from the Bayterek District (*n* = 1/58, 1.7%) (Figure 1). All positive OHFV samples were from the West Kazakhstan region (Figure 1, dark grey area, black circle).

Table 2. Results of OHFV real-time RT-PCR screen in lung tissue of small mammals (*n* = 621) from Almaty region (three trapping sites), Almaty city (seven trapping sites) and West Kazakhstan (19 trapping sites) from 2018/2019. In total, eleven species were collected.

Region	Year	Collected Species	Positive Species (#of positive)
Almaty region	2018/2019	6	0
Almaty city	2018/2019	7	0
West Kazakhstan	2018/2019	6	*A. uralensis* (2), *M. musculus* (2), *C. glareolus* (3)

All other lung tissue samples, as well as all brain tissue samples from small mammals originating from the West Kazakhstan region (2019), Almaty region and Almaty city from 2018 and 2019, were negative for OHFV RNA.

Although it was shown that the selected diagnostic primers identified OHFV with high reliability (Supplemental Table S3), there remains the possibility of a false positive result. A potential cross-reactivity was ruled out by testing both positive human CFS for other closely related flaviviruses such as WNV and TBEV. Both patients did not have any previous infections with either TBEV or WNV [37]. Similarly, all positive OHFV samples originating from ticks were negative for TBEV (data not shown), *Crimean-Congo haemorrhagic fever virus* (CCHFV) [42] and *Rickettsia* spp. [45]. All positive OHFV samples originating from rodents were negative for *Orthohantavirus* and *Rickettsia* spp. (data not shown).

In conclusion, OHFV was, for the first time, detected in human patients, ticks and rodents outside of Russia in the Republic of Kazakhstan.

4. Discussion

OHFV is currently only known to be prevalent in four regions of Russia, namely Kurgan, Tyumen, Omsk and Novosibirsk. Only in these regions, irregular but frequently recurring outbreaks of OHF among the population are recorded [1–3,7]. The Republic of Kazakhstan is adjacent to the known OHFV-endemic areas in Russia—North Kazakhstan, for example, borders directly the regions of Kurgan, Tyumen and Omsk. The major arthropod vector of OHFV is the tick *D. reticulatus*, which was also the tick mainly collected in the regions of North Kazakhstan and Akmola (Table 1). Furthermore, sightings of the muskrat *(O. zibethicus*), the most well-known spreader of OHFV, have been reported along the rivers of Kazakhstan [30]. Since all these mentioned hosts are also endemic to Kazakhstan, it is actually not a question of if but how widespread OHFV is in Kazakhstan.

For this reason, we examined areas of Kazakhstan that are adjacent to the Russian OHFV epidemic areas, such as North Kazakhstan and the Akmola region. In order to establish a cross-sectional study, it was supported by also examining more remote regions such as West Kazakhstan in the west and East Kazakhstan and the Almaty region in the south east of Kazakhstan. Arthropod-borne viral diseases usually have a natural cycle that includes ticks and small rodents as natural reservoirs. The human is usually a dead-end host. In order to draw a complete picture of the distribution of OHFV in Kazakhstan, we screened CSF from human patients with meningitis, pools of ticks and captured small rodents.

The screening on CSF was based on a collection of 130 samples from a previous study about serous meningitis patients of unknown aetiology [37]. However, in this study, only for approximately 20% of the enrolled patients was a causative agent, such as TBEV or WNF, identified. Due to the lack of a reliable OHFV ELISA, the screening had to be performed on viral RNA, although the timeframe during which the patient is viraemic is rather short. Nonetheless, two patients with OHFV showing very weak symptoms were identified, interestingly in Almaty city, far away from the presumed endemic areas in the north of Kazakhstan. Both of the patients did not have a travel history to the north, but one of the positive patients had recently travelled to Kyzylorda in the south. As the OHFV virus is shed by infected animals in a very high titre, it is possible that they became infected by drinking, swimming or washing their hands in contaminated water. Hence, a subsequent screening of rodents and ticks in the area of Almaty city was initiated but did not reveal any positive specimen. However, it should be noted that the number of ticks and rodents examined in the area of Almaty was too small to draw a definite conclusion from our results.

Nevertheless, in other areas, ticks and rodents were indeed positive for OHFV. We identified ticks of the family *Dermacentor* that were positive for OHFV, confirming its important role in the spread of OHFV. Furthermore, we also detected positive pools of *I.*

persulcatus. As already discussed elsewhere, *Ixodes* seems to play a potential role in the sylvatic life cycle of the agent [2,29].

Surprisingly, rodents from West Kazakhstan, a region far away from the known OHFV-endemic regions in Siberia, tested positive for OHFV. It was interesting, however, that all positive rodents were collected in areas close to lakes and rivers. The infected rodents of West Kazakhstan, for example, were collected in the village of Teretiki and around the city of Oral, which is close to the river Ural. Additionally, in Akmola, the positive ticks (*D. marginatus* and *D. reticulatus* and also *I. persulcatus*) were collected in forest steppe areas with many lakes and rivers. In contrast to this, all tick and rodent sampling sites in the region of North Kazakhstan were quite distant from the next river. One could postulate that the spread of OHFV follows the tracks of waterways, since the muskrat has populated rivers in Central Asia since its release there in the 1930s [31,46]. By excreting the virus through their urine and faeces in the water, muskrats infect other (semi)-aquatic small mammals such as *Arvicola amphibius* [3,29]. In addition, OHFV infects other small rodents such as members of *Microtus* spp. [2,29]. Blood-feeding ticks enhance the local distribution of OHFV in new endemic areas [2,3]. Additionally, there is a theory that OHFV has existed unnoticed in Siberia, including Kazakhstan, [3,47], for many decades already and started to cause human casualties only by the introduction of the muskrat. This is opposed by a recently published study that states that OHFV has evolved beyond the TBEV Far Eastern subtype, due to the fact that TBEV has spread in the muskrat population without any involvement of ticks—leading to a new zoonotic agent [48].

In general, information is scarce on the effect of a persistent OHFV infection on its natural hosts. One can assume that all OHFV-positive rodents probably had asymptotic infections, since viral RNA was not detected in their brains. However, laboratory tests propose a fatal neuroinfection [49], and in the wild, *O. zibethicus* is particularly susceptible to acute neuroinfection leading to death [29]. However, other wild vertebrates show mostly asymptomatic diseases with viraemia [3,29].

The question of how OHFV actually came to Kazakhstan will remain the subject of future studies. Due to the fact that human patients are infected in Almaty city also, which is approximately 2000 km away from West Kazakhstan and 500 km away from the Akmola region, it is possible that OHFV is already widespread throughout Kazakhstan. The spread could also have occurred through bird migration, through which infected ticks can travel great distances. Other possibilities include China's Belt and Road Initiative, animal trading, and travelling, whose role in the dissemination of ticks and rodents and potential agents should not be underestimated [50,51].

However, despite this first description of OHFV in Kazakhstan, there is no urgent need for public health measures. OHF has a rather low CFR (0.5–3%) [6], contrary to other endemic zoonotic infections in Central Asia, such as the TBEV Far Eastern subtype with a CFR of 20–30% [52] or CCHFV with a CFR of approximately 30% [53]. Nonetheless, there is a high chance of subclinical or mild cases with only fever and no haemorrhages. This means that infections may remain undiagnosed and undetected [3,7] and any febrile disease can be declared as FUO, which is a noteworthy public health problem [42]. Recently published studies on FUO [34,42,54,55] and a study on serous meningitis in patients in Kazakhstan demonstrate that, in general, there is a need to raise awareness of the effect of emerging zoonoses in the healthcare system of Kazakhstan. There is a need for more reliable diagnostics and an awareness that zoonoses can potentially lead from an initially unspectacular febrile disease to a life-threatening condition. Currently, laboratory diagnosis for OHFV is based on serological methods including an ELISA against OHFV IgM and IgG antibodies [2] or against the OHFV-NS1 antigen [56]. Further detection is performed by seroconversion with paired sera by haemagglutination inhibition, complement fixation test, and neutralisation assays [2]. These tests should be available in all infection hospitals in Central Asia to enable an efficient differential diagnosis.

To complete this interesting evidence of an OHFV distribution in Kazakhstan, the positive samples need to be sequenced. It is also not possible to say into which OHFV

subtype according to Kovalev et al., 2021 [1] the positive samples from Kazakhstan can be classified. Unfortunately, due to the current pandemic and political situation, the sequencing was not possible in this study. However, future investigations including sequencing will show which strains are circulating in wild host animals with regard to the different clusters that seem to exist [1]. Furthermore, a distance analysis will clarify how long OHFV has existed in the regions or whether it has appeared there only recently.

Therefore, it is important to start a systematic cross-sectional and longitudinal study in Kazakhstan including more data of OHFV in patients, rodents and ticks to clarify open questions and to gain a complete picture of OHFV in Kazakhstan—a virus that now officially has left Russian territory and spreads over Central Asia. Future studies will give a deeper insight into the emergence and spreading mechanisms of OHFV all over Kazakhstan.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14040754/s1, Supplemental Table S1: Overview of tick collection. Supplemental Table S2: Overview of small mammal collection. Supplemental Table S3: Testing of primers.

Author Contributions: E.W., L.P. and S.E. conceived the layout of the project. E.W., A.S. (Anna Shin) and N.T. (Nur Tukhanova) performed the laboratory analysis. E.W. performed the statistical analysis and generated the figures and tables. E.W. and L.P. wrote the first draft of the manuscript. A.S. (Alexander Shevtsov), T.N., N.T. (Nurkeldi Turebekov), V.S., A.B., N.M., I.L., Z.S., K.F. and C.E. contributed by providing additional information as well as reviewing the manuscript. S.E. and L.P. supervised the project and oversaw data analysis, manuscript drafting, and revision. All authors have read and agreed to the published version of the manuscript.

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6.2 Tick-borne encephalitis virus and West-Nile fever virus as cause of serous meningitis of unknown origin in Kazakhstan

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ORIGINAL ARTICLE

Tick-borne encephalitis virus and West-Nile fever virus as causes of serous meningitis of unknown origin in Kazakhstan

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Abstract

Flaviviruses are a family of viruses that cause many diseases in humans. Their similarity in the antigenic structure causes a cross-reaction, which complicates the precise diagnostic of disease causing agents. Tick-borne encephalitis virus (TBEV), a member of the flavivirus family, is the cause of tick-borne encephalitis (TBE). Worldwide the awareness of this disease is raising, however, in many countries such as the Republic of Kazakhstan (KZ) there is a lack of serological investigation of flaviviruses in humans. In our study, we focused on two TBE endemic regions of KZ (East Kazakhstan Oblast (EKO) and Almaty (AO)) and a region where TBE cases were registered only since 2010 (Akmola Oblast (AkO)). In KZ, up to 400 cases of serous meningitis of unknown origin were registered annually in the period from 2017 to 2019. Our goals were to calculate the prevalence of antibodies against TBEV in patients with suspected meningitis. We collected 179 sera and 130 cerebrospinal fluid (CSF) samples from patients and included a questionnaire with focus on socio-demographical factors and

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and the National Scientific Center for Highly Dangerous Infections in Almaty, Kazakhstan.

observed tick bites. The human samples were tested with TBEV and West-Nile fever virus (WNFV) IgM and IgG ELISA, by immunofluorescence assay using a flavivirus biochip, and TBEV-specific real-time RT-PCR. We found TBEV and WNFV antibodies in 31 samples by serological and molecular techniques. Seven serum samples out of 31 showed TBEV-specific antibodies, and three serum pairs had WNFV antibodies. Correlating the serological results with the information gained from the questionnaires it becomes apparent that the number of tick bites is a significant factor for a TBEV infection. This result has an impact on diagnostic in KZ and physicians should be aware that both flaviviruses play a role for serous meningitis of unknown origin in KZ.

KEYWORDS
encephalitis, Republic of Kazakhstan, serology, serous meningitis of unknown origin, tick-borne encephalitis virus, West-Nile fever virus

1 | INTRODUCTION

The virus family Flaviviridae comprises about 50 serologically related viruses (Simmonds et al., 2017). This family is globally prevalent and is subject to geographical dispersion by migration of birds, transportation of livestock or mobility in travellers. Human infections with different species of Flaviviridae are often difficult to differentially diagnose by treating practitioners, since their antigenic similarity across species leads to cross reactions in commercially available assays with an increased likelihood of false-positive test results (Rathore & St. John, 2020).

Tick-borne encephalitis virus (TBEV) is one member of the genus *flavivirus* within the family of Flaviviridae. It is a small enveloped virion with a single-stranded positive RNA of about 11 kb length. Five subtypes of TBEV are currently known, the European, the Far-Eastern, the Siberian, the Baikalian and the Himalayan subtype (Dai et al., 2018; Kovalev & Mukhacheva, 2017).

TBEV is the cause of tick-borne encephalitis (TBE), a potentially fatal central nervous system (CNS) infection in humans (Monath, 1990). TBE is endemic in many countries in Europe and Asia and up to 3,000 cases of TBE are annually registered in Europe and up to 10,000 cases in Russia (Süss, 2011). TBEV can either be predominantly transmitted by tick bites from *Ixodes* spp. or *Dermacentor* spp. (Süss, 2011) or more seldom by the consumption of raw milk products (Cisak et al., 2010). About one third of infected and symptomatic patients develop the clinical manifestation of tick-borne encephalitis (Kaiser, 2008) that appears usually in a biphasic presentation. In the first viraemia phase the patient develops nonspecific flu-like symptoms. Only in this phase virus RNA can be detected (Veje et al., 2018). The second phase is characterized by involvement of the CNS with development of potential severe meningitis and meningoencephalitis (Lindquist & Vapalahti, 2008). During this phase the immune system creates first specific IgM and later IgG antibodies against TBEV. The severity of TBE varies from mild to severe with fatal outcomes depending amongst other

Impacts

- Serous meningitis of unknown aetiology is a common symptom among hospitalized patients in Kazakhstan.
- Tick-borne encephalitis virus (TBEV) is a causative agent for many meningitis and meningoencephalitis cases in known TBEV endemic regions and in previously non-endemic regions of Kazakhstan.
- Beside TBEV antibodies also other flavivirus antibodies such as those against West-Nile fever virus (WNFV) were identified in Kazakhstan.

reasons on age and viral subtype (Kaiser, 2008). The mortality rate in different regions ranges from 1% to 20% depending on the TBEV subtype present in the region (Barrett et al., 2008). TBE may also develop into a long-term sequela (Veje et al., 2016) that include residual neuropsychological symptoms, headache, ataxia, paresis and muscle atrophy (Karelis et al., 2012).

West-Nile fever virus (WNFV) is another member of the Flaviviridae family and is the causative agent of West-Nile fever that can develop encephalitis. The main vector of WNFV are mosquitoes, but transmissions by blood transfusion, organ transplantation and laboratory incidents are also possible. The clinical presentation of WNF resembles that of TBE with the first phase of unspecific symptoms such as fever and myalgia and the development of encephalitis in a second phase with a potentially fatal outcome (Colpitts et al., 2012).

The Republic of Kazakhstan (KZ) is located in Central Asia with a diverse geography and climate (Peintner et al., 2021). KZ is subdivided into 14 administrative regions called oblasts and three major cities. In the North it is bordering to the Russian region of Western Siberia that is endemic for TBE (Figure 1a). The total population of KZ is 18.8 million, with 42.3% living in rural areas. Tick

bites are frequent in the rural Kazakhstan population. However, in a period of ten years (2011–2020) only 363 TBE cases were registered in KZ (Table 1, Figure 1b) (NCPHC, 2011). Officially there are three TBE endemic regions in KZ namely Almaty Oblast (AO), East Kazakhstan Oblast (EKO) and Akmola Oblast (AkO). Moreover, in a recent study we could show that TBEV detected in collected ticks in Almaty Oblast, a region south-east of Kazakhstan, belong to the Siberian subtype (Abdiyeva, 2020). The first confirmed TBE cases in AkO were reported in 2010, leading to the declaration of AkO as an endemic region only in 2018. There were so far no reported TBE cases in the northern parts of KZ since initial studies in 1964 (Kereyev, 1965).

The reasons for the scarcity in epidemiological data on TBE in KZ is manifold and comprise unspecific symptomatology, lacking awareness in physicians and non-availability of testing capacity. According to the National Centre of Public Health Care of the Ministry of Health of the Republic of Kazakhstan (NCPHC, 2011) up to 400 cases of serous meningitis with unknown origin were registered annually in the time period 2017–2019, of which a part may be presumed to be unrecognized TBE cases (NCPHC, 2011).

This study was thus implemented with the aim to assess the role played by TBEV in cases of meningitis of unknown origin in Kazakhstan regions of EKO, AkO and Almaty city. Furthermore, we aimed to describe the socio-demographical and medical characteristics of the patients from whom samples were collected.

2 | MATERIALS AND METHODS

2.1 | Study setting and sample collection

This study was set up as a cross-sectional study involving individuals with a clinical suspicion of meningitis. The investigations were performed in eight hospitals of three regions, in East Kazakhstan, Akmola and Almaty city from April to October (TBE endemic seasons) in the years 2018 and 2019. The study was conducted upon ethical approval of the Kazakh National Medical University (opinion number # 565) and the Ludwig-Maximilians-Universität (opinion number #19-373) ethics committees. A suspected case of meningitis/meningoencephalitis was defined as any patient with fever and the presence of persistent headache and/or meningeal signs. Additional inclusion criteria were headache and/or nausea, vomiting and unconsciousness, while exclusion criteria were age below 18 years and mental conditions such as psychosis or uncontrolled depression. All participants were required to sign an informed consent. Upon inclusion participants were administered a questionnaire and then paired serum samples at the date of hospital admission and two weeks later and cerebrospinal fluid (CSF) were collected. Serum samples and CSF were stored at −20°C until further analysis. Only patients where the two sera were available were included in the study. The absence of CSF or an incomplete questionnaire was not considered an exclusion criteria.

2.2 | Questionnaire

The paper based self-administered questionnaire, upon participant request supported by hospital personnel, was covering data on socio-demographical characteristics, living and housing, travelling history, contact to livestock, vector habitat factor, observations of tick bites, clinical symptoms and vaccination status. The data was collected on paper forms and then entered into a Stata-based database for further analysis (StataCorp, LLC, Texas, USA).

2.3 | ELISA analysis

Serum samples were analysed for TBEV-specific IgG and IgM using a commercial ELISA kit as described by the manufacturer's instructions (Anti-TBEV IgG/M ELISA; Euroimmun, Luebeck, Germany).

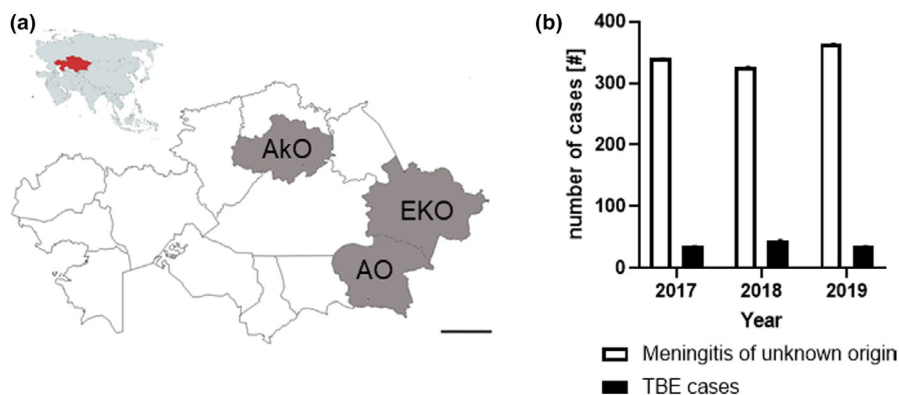

FIGURE 1 Overview on the situation of flavivirus infections in the Republic of Kazakhstan. (a) All patient samples were collected in three oblasts of the Republic of Kazakhstan, a country located in Central Asia (small map). The three oblasts were Akmola Oblast (AkO), Eastern Kazakhstan (EKO) and Almaty Oblast (AO). Size marker = 500 km. (b) Total published numbers of reported cases of serous meningitis in Kazakhstan in the years from 2017 to 2019. Many cases of serous meningitis have an unknown origin (white bar). A tick-borne encephalitis virus infection induced meningitis is also frequently diagnosed (black bar)

TABLE 1 Tick-borne encephalitis cases in KAZ 2011–2020 (Modified from NCPHC. 2011–2020: Annual report about separate infectious and parasite diseases of the population of the Republic of Kazakhstan)

	Tick-borne encephalitis cases per year										
Administrative territories	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	Total
Akmola oblast	3	3	0	4	6	2	8	3	3	6	38
Almaty oblast	6	5	6	8	10	12	4	11	7	1	70
East Kazakhstan oblast	20	13	13	5	15	21	17	22	15	21	162
South Kazakhstan oblast	1[a]	0	0	0	0	0	0	0	0	0	1
Almaty city	10	12	8	11	6	12	6	5	5	0	75
Nur-Sultan city	0	0	0	0	0	0	0	0	1	0	1
Zhambyl oblast	0	0	0	0	1[a]	0	0	0	0	0	1
Kostanay oblast	0	0	0	0	2[a]	0	0	1[a]	0	0	3
Pavlodar oblast	0	0	0	0	0	1[a]	0	0	0	0	1
North Kazakhstan oblast	0	0	0	0	0	0	0	3	4	4	11
Total	40	33	27	28	40	48	35	45	35	32	363

[a]Imported cases of TBE from other oblasts\countries.

Test results were expressed in relative units per millilitre (RU/ml). A semi-quantitative method for IgG was used with a calibrator value of 2 (corresponding to 20 RU/ml) following the recommendations for calculating the ratio as the optical density of the positive control divided by the optical density of the calibrator value 2. Ratio results below 0.8 were interpreted as negative, from 0.8 to 1.1 as indeterminate and above 1.1 counted as positive.

The investigation of Anti-TBEV IgM (Anti-TBEV IgM ELISA; Euroimmun, Luebeck, Germany) by ELISA was performed similarly as stated above using a semi-quantitative method with a standard calibrator and the same calculation procedure according to the manufacturer's instructions.

The paired first and second serum samples were analysed for anti-TBEV IgG starting from the second serum. If the sample was positive then the first serum was analysed. Only when both sera were tested positive a titration was performed. If there was at least a fourfold increase of titres between first and second serum, an acute infection was assumed. All first and second sera were screened for Anti-TBEV IgM. Samples positive for IgM and IgG in ELISAs were investigated further as described below in an Immunofluorescence assay (IIFA).

To screen for WNFV an Anti-WNFV IgM ELISA was performed with an assay by a different manufacturer (VectoBest, Novosibirsk, Russia) according to the manufacturer's instructions. The Anti-WNFV IgG investigation (Anti-WNF IgG ELISA; Euroimmun, Luebeck, Germany) was performed similar to the anti-TBEV IgG screen. The first and second sera were probed for anti-WNFV IgM and IgG with a subsequent titration step as it was described above with TBEV. The WNFV positive samples were used for further testing in the IIFA.

Furthermore, the first sera positive for TBEV and WNFV antibodies were further checked for anti-Cytomegalovirus (CMV) and anti-Epstein-Barr virus (EBV) IgM with ELISA (Euroimmun, Luebeck, Germany) according the kit instructions to exclude false-positive results.

2.4 | Immunofluorescence assay

All sera reacting TBEV positive in ELISA were additionally probed using an immunofluorescence assay (IIFA) (Euroimmun, Luebeck, Germany) with a better specificity profile as compared to the ELISA assays, to exclude potential cross-reactivity with antibodies produced by patient's exposure to other flaviviruses. Herein sample dilutions of sera were 1:10, 1:100 and incubated with the EU 14 cells covered slides following the instructions by the manufacturer. The titre is defined as the sample dilution factor for which specific fluorescence is visible. Samples were checked for IgM and IgG with four flaviviruses TBEV, WNFV, YFV (Yellow Fever virus) and JEV (Japanese encephalitis virus). The biochip analysis was performed on a MicroOptix MX 300 fluorescence microscope using 40x magnification.

In this study a TBEV/WNFV infection was counted when in the respective ELISA screenings at least one of the paired sera was positive for IgM or IgG and as well a positive reaction for IgM/IgG antibodies in the IIFA screen.

2.5 | Nuclear amplification technique

The nuclear amplification analysis was set up as a three-step procedure. Viral RNA extraction from all samples of CSF and first serum was carried out with the QiAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). In a first step, RNA extracted from serum and CSF were tested by real-time RT-PCR on a Rotor-Gene Q device (Schwaiger, 2003) with the QIAGEN QuantiTect Virus Kit for TBEV and Omsk haemorrhagic fever virus (OHFV) (Růžek, 2010). The samples tested positive in the first step were analysed in a second step by conventional RT-PCR, where the TBEV target was the E-gene (1687bp) (Forward primers: RSSE 947A+RSSE 947B: 5'-TCC TCT GCC TGG CTC CGG TTT ATG-3 + 5'-TCT TGT GCC TGG CTC

CGG TTT ATG-3' and the reverse primer RSSEc2579: 5'-CCT GGC GTT TCT GGG TAG TAT G-3'). Amplification was performed in 45 cycles with an annealing temperature of 52°C, using the Invitrogen™ SuperScript™ III One-Step RT-PCR System and PCR products were visualized on a 1.5% agarose gel.

In a third step, those samples that revealed positive results in both the first and second step were to be sequenced by the Sanger method with the ABI Prism Big Dye Terminator V3.1 Cycle Sequencing Kit and 3500xl Genetic Analyser machine, using the initial primers of the RT-PCR amplification.

2.6 | Hospital in-house analyses

As part of the routine investigation, patients were analysed by the hospitals for TBEV infection (in addition to the study related analyses), borreliosis and mumps by serology, for enterovirus infection by molecular biology and for bacterial infections such as staphylococcus or streptococcus by standard bacteriological screening. However, all collected sera were again tested for TBEV as per the study protocol, even if they were positive in the routine investigations.

2.7 | Variables and statistical analysis

All socio-demographical and symptomatic characteristics of the involved participants were presented in absolute numbers and percentages, which are cross tabulated for presence and type of Flaviviridae detected. The prevalence of IgM and IgG detected with ELISA immune fluorescence is also presented in absolute numbers and percentages for TBEV and WNFV, respectively, as well as an overview of the diagnostic pattern of all confirmed TBEV and WNFV diagnosis are cross-referenced with the hospital diagnosis. Furthermore, we used binomial logistic regression to identify potential factors that drive an infection with Flaviviridae. First the probability of one factor causing an infection was calculated in Odd Ratios (OR) with a 95% confidence interval (CI). This unadjusted OR were then adjusted for each other's effect in a multivariate model with significance (p-value) set at 0.05. Statistical analysis was performed using Stata 2015 (StataCorp, LLC, Texas, USA).

3 | RESULTS

A total of 179 patients with suspected cases of meningitis from eight hospitals in three regions (Table 2) were enrolled in this study. However, for thirteen patients with suspected meningitis there was only first serum available and so they could not be included in the study, as they did not fulfil all inclusion criteria. This were six samples from Almaty, six samples from East Kazakhstan and one sample from Akmola region.

Therefore, the total number of samples investigated in this study is 166. Furthermore, from 130 patients CSF was collected.

TABLE 2 Overview of collected samples in patients with suspected case of meningitis/meningoencephalitis in three regions of Kazakhstan collected from April 2018 to October 2019. For all further investigations all samples with first and second serum were included in the study ($n = 166$)

Region/hospital	Collected samples		
	First serum	Second serum	CSF
Almaty (AO)			
City infectious hospital	153	147	115
East Kazakhstan (EKO)			
Oskemen	9	5	7
Ridder	2	2	0
Altay	4	3	1
Katon-Karagay	1	0	0
Akmola (AkO)			
Kokshetau	4	4	2
Shuchinsk	4	3	3
Sandyktau	2	2	2
Total	179	166	130

3.1 | Correlation analysis of serous meningitis patients with socioeconomic factors

Main symptoms as reported in the questionnaire were fever ($n = 145/178$, 81.5%), headache ($n = 171/178$, 96.1%) and neck pain ($n = 80/178$, 44.9%) (Table 3). One patient was previously vaccinated against TBEV in July 2019 and received anti-TBEV-specific IgG as post-exposure prophylaxis after a tick bite in September 2019. None of the patients had a vaccination against other flaviviruses such as Japanese Encephalitis Virus or Yellow Fever Virus. Most of the patient samples were collected in Almaty city (85.5%, $n = 153$), in East Kazakhstan (8.9%, $n = 16$) and in Akmola (5.6%, $n = 10$) (Figure 1a). Male gender prevailed with 60.9% ($n = 109$) and the mean age of the patients was 28 ($SD \pm 11$) years. On 50 patients the hospitals performed routine laboratory tests, including bacteriological methods conducted in different types of samples, as well as agent specific PCR assays. In detail, for 28 patients (15.6%) an acute meningitis caused by *Enterovirus* was confirmed, for ten patients a meningitis caused by *Neisseria meningitidis* was diagnosed (5.6%), four patients had Human immunodeficiency virus in their serum (2.2%), one patient suffered from *Streptococcus pneumoniae* (0.6%), one from *Staphylococcus sp.*, one was positive for a mumps virus, one patient carried *Mycobacterium tuberculosis*, and one patient contained larval cysts of the parasite *Taenia solium*. Regarding to the TBEV endemic season 65% of patients were screened for TBEV immediately after hospitalization using a Vector Best IgM/IgG ELISA. For six samples an infection with TBEV was diagnosed by the hospitals as the IgM ELISA was positive.

For 63.6% ($n = 49/77$) of the patients a previous trip into nature was recorded and a tick bite was noticed in 7.8% ($n = 6/77$) of the patients. Consumption of raw milk/milk products was described in 4% ($n = 7/177$) (Table S1). Most patients lived in urban

TABLE 3 Clinical symptoms registered in patients with suspected cases of meningitis in three regions of Kazakhstan collected from April 2018 to October 2019

Symptom	TBE (% of symptomatic patients that were confirmed for TBE)	WNF (% of symptomatic patients that were confirmed for WNF)	Flaviviridae (% of symptomatic patients that were confirmed for any flavivirus)	Negative for Flaviviridae (% of symptomatic patients that were negative for any flavivirus)	Total (% of all enrolled patients that presented the symptom) *N* = 178
Fever	8 (5.5)	2 (1.4)	10 (6.9)	135 (93.1)	145 (81.5)
Headache	10 (5.8)	2 (1.2)	12 (7)	159 (93)	171 (96.1)
Neck pain	6 (7.5)	1 (1.3)	7 (8.8)	73 (91.3)	80 (44.9)
Odynophagia	0 (0)	1 (4.8)	1 (4.8)	20 (95.2)	21 (11.8)
Arthralgia	2 (5.3)	0 (0)	2 (5.3)	36 (94.7)	38 (21.3)
Stomach pain	0 (0)	0 (0)	0 (0)	5 (100)	5 (2.8)
Back pain	2 (7.1)	0 (0)	22 (7.1)	26 (92.9)	28 (15.7)
Earache	1 (6.7)	0 (0)	1 (6.7)	14 (93.3)	15 (8.4)
Cough	0 (0)	1 (7.7)	1 (7.7)	12 (92.3)	13 (7.3)
Difficulty with speaking, hearing, seeing	2 (12.5)	0 (0)	2 (12.5)	14 (87.5)	16 (9)
Seizures	0 (0)	0 (0)	0 (0)	3 (100)	3 (1.7)
Breath difficulty	0 (0)	0 (0)	0 (0)	5 (100)	5 (100)
Rapid breath	1 (12.5)	0 (0)	1 (12.5)	7 (87.5)	8 (4.5)
Sore throat	0 (0)	0 (0)	0 (0)	24 (14.5)	24 (13.5)
Nose congestion	1 (4.3)	0 (0)	1 (4.3)	22 (95.7)	23 (12.9)
Lymphnodes	0 (0)	0 (0)	0 (0)	5 (100)	5 (2.8)

Note: NB: Total number of participants in this table sum up to 178 due to missing clinical data from one participant.

TABLE 4 Prevalence of IgG and IgM antibodies against flaviviruses as established by (a) ELISA and (b) immune fluorescence in patients with suspected case of meningitis/meningoencephalitis in three regions of Kazakhstan collected from April 2018 to October 2019[a]

(a) ELISA results

Region	TBEV IgM ELISA positive (%)	TBEV IgG ELISA positive (%)	WNF IgM ELISA positive (%)	WNF IgG ELISA positive (%)
Almaty (*n* = 147)	12 (8.2)	7 (4.8)	5 (3.4)	4 (2.7)
East Kazakhstan (*n* = 10)	3 (30)	3 (30)	0 (0)	1 (10)
Akmola (*n* = 9)	4 (44.4)	2 (22.2)	1 (11.1)	1 (11.1)
Total (*n* = 166)	19 (11.4)	12 (7.2)	6 (3.6)	6 (3.6)

(b) Confirmation of ELISA positive results with IIFT

	IIFT IgM (1:10, 1:100) (%)				IIFT IgG (1:10, 1:100) (%)			
Region	TBEV	WNFV	JEV	total	TBEV	WNFV	JEV	Total
Almaty (*n* = 147)	7 (4.8)	6 (4.1)	0	13 (8.8)	3 (2)	3 (2)	0	6 (4)
East (*n* = 10)	3 (30)	0	0	3 (30)	3 (30)[a]	1 (10)[a]	1 (10)[a]	5 (50)
Akmola (*n* = 9)	0	0	0	0	1 (11.1)	0	0	1 (11.1)
Total (*n* = 166)	10 (6.02)	6 (3.6)	0	16 (9.6)	7 (4.2)	4 (2.4)	1 (0.6)	12 (7.2)

Abbreviations: JEV, Japanese encephalitis virus; TBEV tick-borne encephalitis virus; WNFV, West-Nile fever virus.
[a]One patient received TBEV-specific IgG for treatment and emergency prophylaxis.

area (87.2%, *n* = 156/179) or lived in an area with dense vegetation (54%, *n* = 95/176). About 9.1% (*n* = 16/176) reported to live in the vicinity of grassland.

As concerns factors associated to the diagnosis of any Flaviviridae (Table S2), we performed a logistic regression analysis to identify a correlations of a flavivirus infection with

socioeconomic factors. Variables are age, sex, region, a recent trip to endemic areas, a raw milk product consumption, residence in an urban or rural living area, contact with cats, dogs or birds, recorded tick bites and vegetation around the house. When adjusted in a multivariate model, it reveals that persons who reported to have had repeatedly tick bites had significantly higher chances of being flavivirus test-positive ($p < .005$). Two other factors, however, without any significance when adjusted for other covariates but worth taking note of in the univariate analysis, are the age ($p = .03$) and region of residence of the patient ($p = .022$). An increasing age resulted in growing chances of a flavivirus confirmation, and likewise higher chances when living in East Kazakhstan, Akmola, Russia and 'Other Regions' as compared to living in the region around Almaty city (Table S2).

3.2 | Serological analysis of sera and CSF samples

19 out of the 166 samples were positive for IgM antibodies (11.4%) either in the first and/or second serum (Table 4a). Out of these IgM positive samples eight out of 166 (4.8%) were positive for TBEV IgM in the first and second serum of the paired sera. A subsequent titration of the sera to estimate the antibody content in these eight sera yielded comparable titres between first and second serum.

Further, twelve samples (7.2%) were positive for TBEV IgG antibodies. From those twelve, in six out of 166 (3.6%) samples IgG was detected in the paired first and second serum. Again, a serum titration of these six serum pairs revealed similar titres.

In summary 31/166 (18.7%, IgM/IgG) TBEV positive samples were detected using ELISA as screening method. Nine (5.4%) of them had solitary IgM antibodies in the first serum but not in the second serum. Two sera (1.2%) were only IgM positive in the second serum but not in the initial sampling.

From the 31 TBEV IgM/IgG ELISA positive samples 17 showed a reactivity with flaviviruses on an IIFA biochip (Figure 2). TBEV IgG was confirmed in six (3.6%) samples and IgM in ten (6.02%) samples by IIFA (1:10, 1:100) (Table 4b). Five samples had both types of immunoglobulin (IgM/IgG) (Figure 2a and b).

West-Nile fever virus (WNFV) IgM antibodies were revealed in six (3.6%) tested IIFA samples and WNFV IgG in four (2.4%) of the samples (Figure 2b). Two samples had both, IgM and IgG WNFV antibodies in the flavivirus biochip.

Intriguingly, a serum pair from one patient (OSK 7) with a suspected case of meningitis reacted with four viruses in the IIFA (Table 5). Beside a positive signal for TBEV and WNFV, this patient was also reactive for Japanese Encephalitis Virus (JEV) and Yellow fever virus (YFV) in serum dilutions from 1:10 till 1:400 (Figure 2c and d).

A further screening for other viruses as recommended by the manufacturer of the ELISA revealed that one TBEV antibody positive sample also reacted positive for EBV IgM and two samples replied in the CMV IgM ELISA. Interestingly, one patient (ALM 28) was positive for four acute infections that are TBEV IgM, WNFV IgM, EBV IgM and CMV IgM, a result that has to be handled with care.

3.3 | Molecular biological analysis of CSF samples

In order to detect TBEV RNA, cerebrospinal fluid (CSF) was screened for viral RNA by RT-PCR. For 130 of the 166 patients CSF specimen were available. For the other 36 patients CSF samples were missing due to difficulties performing the spinal puncture. Two of those 130 CSF samples (1.5%) were positive in the first step TBEV-specific real-time RT-PCR. However, this could not be confirmed using conventional RT-PCR targeting the E-gene of TBEV. Though, these two CSF samples from patients with suspected cases of meningitis were positive in the serological investigation for an acute TBEV infection.

To sum up our findings, in Almaty region we found six sera from patients reactive indicating two TBEV acute infections, one previous TBEV infection, two WNFV acute infections and one previous WNFV infection. In the East Kazakhstan region three positive samples were detected resulting in two TBEV acute infections and one previous TBEV infection. In Akmola region was one reactive serum pair indicating an acute TBE infection. All seven TBEV positive patients, either with a previous or acute infection, were male and for WNFV one out of three patients was male. A tick bite was registered in three cases and none of these ten patients was vaccinated against TBEV, but one got anti-TBEV-specific IgG after the observed tick bite. Details of the ten flavivirus positive patients are summarized in Table 5.

4 | DISCUSSION

This study was conceived in order to investigate the potential role of TBEV in patients with suspected cases of serous meningitis of unknown aetiology in Kazakhstan. The Central Asian country has a considerable number of meningitis of unknown origin meandering around 350 cases per year (Figure 1b). In 2018 in KAZ 326 and in 2019 364 cases of serous meningitis with unknown origin were registered, mostly in the three TBEV endemic areas in Kazakhstan (NCPHC, 2011). The onset of TBE is nonspecific (Lindquist & Vapalahti, 2008; Ruzek et al., 2019; Yoshii et al., 2017), and it is, therefore, difficult for clinicians to arrive at a differentiated diagnosis, especially when a tick bite is not recalled or reported by the patient. Furthermore, laboratory assays for TBE are not widely available in KZ and are in most cases mainly performed as a serological screening of a single serum sample. Due to the unspecific presentation with varying degrees of severity of TBEV infections (from unspecific, mild to severe forms, (Kaiser, 2008)) there is the suspicion that TBE is largely under-reported in KZ. To address these issues a multi-centre study was initiated to screen for TBEV in patients with suspected cases of serous meningitis. In total 179 suspected cases

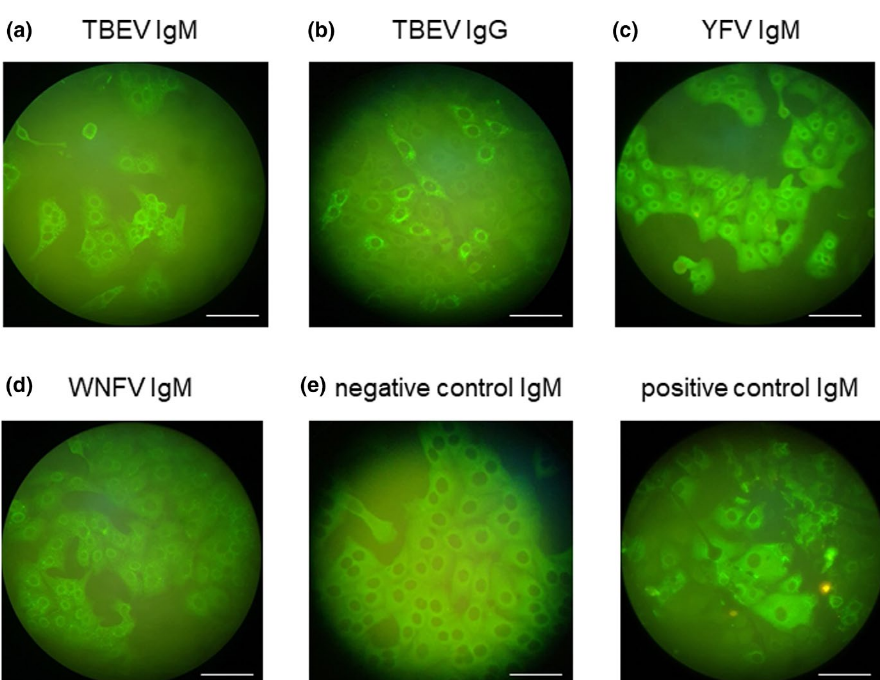

FIGURE 2 Representative images of the indirect immune fluorescence assay (IIFA). All ELISA positive sera were tested on an IIFA biochip to reveal any potential cross reactions by closely related other viral infections. Positive staining is shown for (a) TBEV IgM, (b) TBEV IgG, (c) YFV IgM and (d) WNFV IgM. (e) The analysis was calibrated using negative and positive control images to set the system. Magnification = 40x, size bar = 50 μm

of serous meningitis were initially included in the study. The biggest part of samples was collected in Almaty city and it is important to mention that in 2018 there was a meningococcal meningitis outbreak in Almaty (NCPHC, 2011). Samples that included first and second sera were the basis for this serological study, further for many patients also CSF was available. Among the 166 included patients, ten patients were already recorded as an acute TBE by the hospitals, since TBEV IgM was detected by Vector Best ELISA. However, we were only able to confirm five of them. This is probably because of differences in the sensitivity and specificity of different commercial kits, as has been reported elsewhere (Reusken et al., 2019).

Differential diagnosis by serological methods face the problem that antibodies against Flaviviridae are highly cross-reactive. The gold standard for differentiating flavivirus antibodies is to compare ELISA and IIFA results, and to confirm it by species specific neutralization assays. However, the latter is not established in Kazakhstan (Rathore & St. John, 2020). Further, in the recent years several specific NS1 IgG antibody ELISAs have been developed. NS1 IgG antibody ELISA was used to distinguish between infection induced and vaccine induced antibodies (Girl et al., 2020). A multi-method analysis of all available samples from this study was confirmed as acute TBEV infection in five patients and previous infections in two patients. Some patients had quite unique infection histories and screening results. For instance, in one sample we yielded high levels of TBEV and WNFV IgG in both sera on ELISA, and the IIFA showed a reaction with TBEV IgM. But in the IIFA IgG screen this sample was positive for TBEV, WNFV, JEV and YFV. By looking on the patient history, we saw, that this patient was born and grew up in East Kazakhstan, later he moved to Brazil for several years. We may suspect that this patient had previous TBEV infection potentially dating back years, and acquired later during his stay abroad immunological remnants of exposure to other flaviviruses such as Dengue. Dengue is reported to have cross-reactivity with JEV, YFV and WNFV (Boyd et al., 2018).

A further interesting patient had a clinical manifestation of a classical for serous meningitis, with symptoms of high fever, neck pain and headache developing after a tick bite. In our serological diagnosis he was positive for TBEV IgM and IgG in both sera and in the IIFA a strong IgG signal could be detected. This patient further got treated by a single dose injection with human anti-TBEV-specific immunoglobulin. This treatment is uniquely used as an emergency post-exposure prophylaxis against TBE during the first three days after a tick bite (Olefir et al., 2015) and it is mainly used in Russia, Belarus and Kazakhstan.

Historically the oblast of East Kazakhstan had the highest reported TBE incidence in Kazakhstan. After a mass anti-TBEV vaccination campaign of its population in 2016 the incidence decreased. Now in East Kazakhstan most new infections reside in patients that newly moved to this oblast from other regions. However, we also find some patients where the vaccination with available Russian TBEV vaccines failed, which is also reported from other countries with other TBEV vaccines (Dobler et al., 2020). For instance, a patient had the first dose of vaccination and two months later he was bitten by a tick. He immediately received specific anti-TBEV immunoglobulin. Due to the clinical symptoms and the initial laboratory screening in hospital he was officially registered as a TBE case. However, all our various methodical approaches failed to find any anti-TBEV antibodies. This is surprisingly, since the patient got vaccinated and on top received anti-TBEV immunoglobulin and hence should have some reactive antibodies against TBEV.

Two further patients from Almaty city were highly interesting cases. Those two patients presented at the infectious disease hospital with serous meningitis of unknown origin. Both those patients were negative in the first and second serum on IgM and IgG ELISA.

TABLE 5 Overview of 10 ELISA TBEV and WNFV positive samples that were confirmed by IIFT/PCR

	TBEV ELISA serum		TBEV IIFT serum		WNFV ELISA Serum		WNFV IIFT serum						
Sample ID	**IgM**	**IgG**	**IgM**	**IgG**	**IgM**	**IgG**	**IgM**	**IgG**	**CSF PCR**	**Tick bite**	**AntiTBE Ig[a]**	**Hospital diagnosis**	**Final diagnosis**
ALM 126	–	±	+	–	–	–	–	–	+	–	–	MUO	TBE acute infection
ALM 137	–	–	+	–	–	–	–	–	+	–	–	MUO	TBE acute infection
GLU 9	+	+	+	+	–	–	–	–	–	+	–	TBE	TBE acute infection
GLU 13	+	+	+	+	–	–	–	–	–	+	–	TBE	TBE acute infection
KOK 20	+	+	–	+	–	–	–	–	–	+	+	MUO	TBE acute infection
OSK 7	–	+	±	+	–	+	–	+	–	–	–	cysticercosis	Previous TBE infection
ALM 52	–	+	+	+	–	–	–	–	–	–	–	Enterovirus	Previous TBE infection
ALM 23	+	+	+	–	±	+	+	+	–	–	–	MUO	WNF acute infection
ALM 80	–	+	–	–	+	+	–	+	–	–	–	MUO	WNF acute infection
ALM 53	–	–	–	–	–	–	±	+	–	–	–	TBE	Previous WNF infection

Abbreviations: ALM, Almaty city; CSF, cerebrospinal fluid; GLU, Glubokovskiy district; KOK, Kokshetau city; MUO, meningitis of unknown origin; OSK, Oskemen city; TBE, tick-borne encephalitis; TBEV, tick-borne encephalitis virus; WNFV, West-Nile fever virus.

[a]AntiTBE Ig—human immunoglobulin against tick-borne encephalitis (titre of haemagglutinating antibodies to tick-borne encephalitis virus not less than 1:80), use for TBE treatment and emergency prophylaxis.

However, the IIFA assay reacted positive in both sera on IgM. It is worth noting, that during a TBEV infection, IgM antibodies only appear approximately five days after the debut of the infection in blood and CSF and detection of TBEV RNA in CSF is at the same time rarely successful (Roelandt et al., 2017). Since these patients had IgM levels below the sensitivity level of the ELISA, we suspected an acute TBEV infection. Indeed, after performing a TBEV-specific real-time RT-PCR on the CSF of both patients, viral RNA was detected. Unfortunately, levels of the viral RNA in the CSF was too low to grow the respective virus in cell culture or to partially sequence it.

Routine exposure to tick bites has been shown many studies to be the main risk factor of the TBE infection (Imhoff et al., 2015). The results of this study do not only go further to strengthen that point but also bring out the need to estimate vector concentration within different regions of the country, even those not yet endemic to Flaviviridae. As a consequence, appropriate mechanisms for vector control should be put in place. Mass communication for general hygiene and behavioural change should be increased. In some samples our array of assays also showed its limitations in terms of validity. For instance, in the patient from Almaty in whom the ELISA results were positive for the IgM antibodies for infections with TBEV, EBV, CMV and WNFV. However, further in-depth analysis with IIFA only showed a weak result for IgM that fully disappeared upon further serum dilution. Since it is highly improbable that the patient suffered from four acute infections simultaneously, it has to be considered that some other factors caused a false-positive reaction of the ELISA screen. For instance, it is known, that a high serum level of rheumatoid factor may give an unspecific assay reaction (Verkooyen et al., 1992). In addition, it is possible that the patient carried some other unrelated virus or bacterium we did not check for in our panel that unspecific reacts with the virus assays we employed. Due to this high level of uncertainty and the low titre for TBEV in the IIFA, we classified this patient as negative for TBEV.

Finally, two samples were shifted to another study that run in parallel to this examination since many hints lead to the suspicion that those patients actually suffered from an infection with Omsk Haemorrhagic Fever (OHF), but only after an TBEV infection was excluded (Wagner et al., accepted in Viruses). At the moment, there is no official WNFV registration in Kazakhstan, but according to a NSCEDI study (Maikanov & Ayazbaev, 2016), we know that WNFV was detected in West Kazakhstan in mosquitos as well as WNFV-specific IgG antibodies were detected in 5.4% West Kazakhstan Oblast population. In one case in our study we could confirm WNFV infection through a positive IgM assay, and as a consequence correct the hospital diagnosis of a suspected TBEV infection. With this study we are able to corroborate occurrence of WNFV infections in Almaty region. This increase in evidence for the endemic character of WNFV in Kazakhstan should call for increased attention to this disease entity by medical staff, along with procurement of diagnostic capacities for WNFV detection.

In summary, both the flaviviruses TBEV and WNFV were confirmed in ten samples from suspected cases of serous meningitis originating from three oblasts in KZ. Five samples showed a constellation of an acute TBEV infection and two samples that of a previous TBEV infection. In addition, two samples revealed the constellation of an acute WNFV infection, and one sample that of a previous WNFV infection. Some of the patients were identified in regions that are not officially declared as endemic areas. In non-endemic areas medical staff and diagnostic laboratories are often not able to faithfully diagnose such infections. Increased efforts in awareness raising could raise the levels of detected infections and lower the number of serous meningitis with unknown aetiology in Kazakhstan.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors declare that there is no financial or personal relationship with other people or organisations that could inappropriately influence the work. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by Bundeswehr Joint Medical Service or any other governmental institutions.

AUTHOR CONTRIBUTIONS

AS, LP and SE conceived the layout of the project. AS and JJN performed statistical analysis and generated the figures and tables. AS and SE wrote the first draft of the manuscript. NT, JJN, KA, NT, ZS, RY, SA, LY, LM, TN, YS, GF, MH, EW contributed providing additional information as well as reviewing the manuscript. SE and LP supervised the project as well as oversaw data analysis, manuscript drafting and revision.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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6.3 Molecular characterisation and phylogeny of Tula virus in Kazakhstan

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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.

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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).

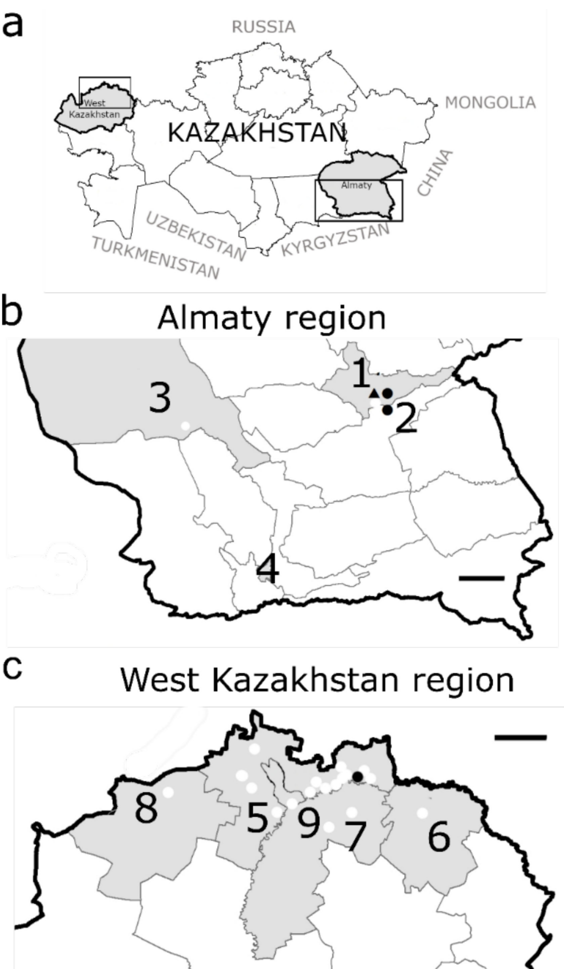

Figure 1. Geographical location of the sampling points for small mammals in Kazakhstan. (**a**): Kazakhstan is divided in 14 oblasts (=regions) and located in Central Asia. (**b**): Almaty region and Almaty city: **1.** Tekeli city: 2 trapping sites; **2.** village Rudniychniy: 1 trapping site; **3.** 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′-TGATGCATACTG TGTGCAAAC-3′, 1c-fw: 5′-CAGTATGATGCATACTGTGTCCAA-3′, 1d-fw: 5′-TGATG CCTATTGTGTTCAGAC-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).

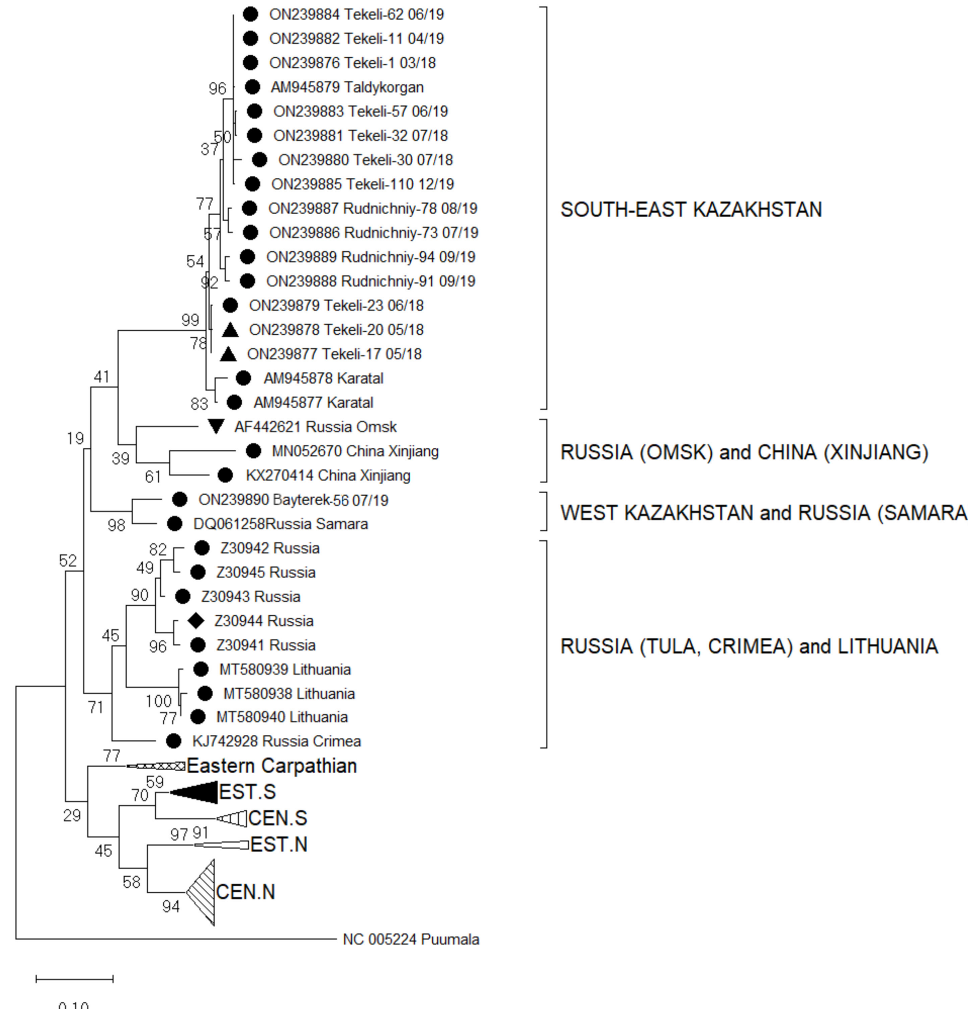

Figure 2. Phylogenetic analysis by Maximum Likelihood method of the S segments (346 nucleotides (nt), positions of sequences 715–1061 nt in regard to the reference sequence AM945879) of Tula virus in Kazakhstan. The tree with the highest log likelihood (−5756.38) is shown. The percentage of trees in 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*.

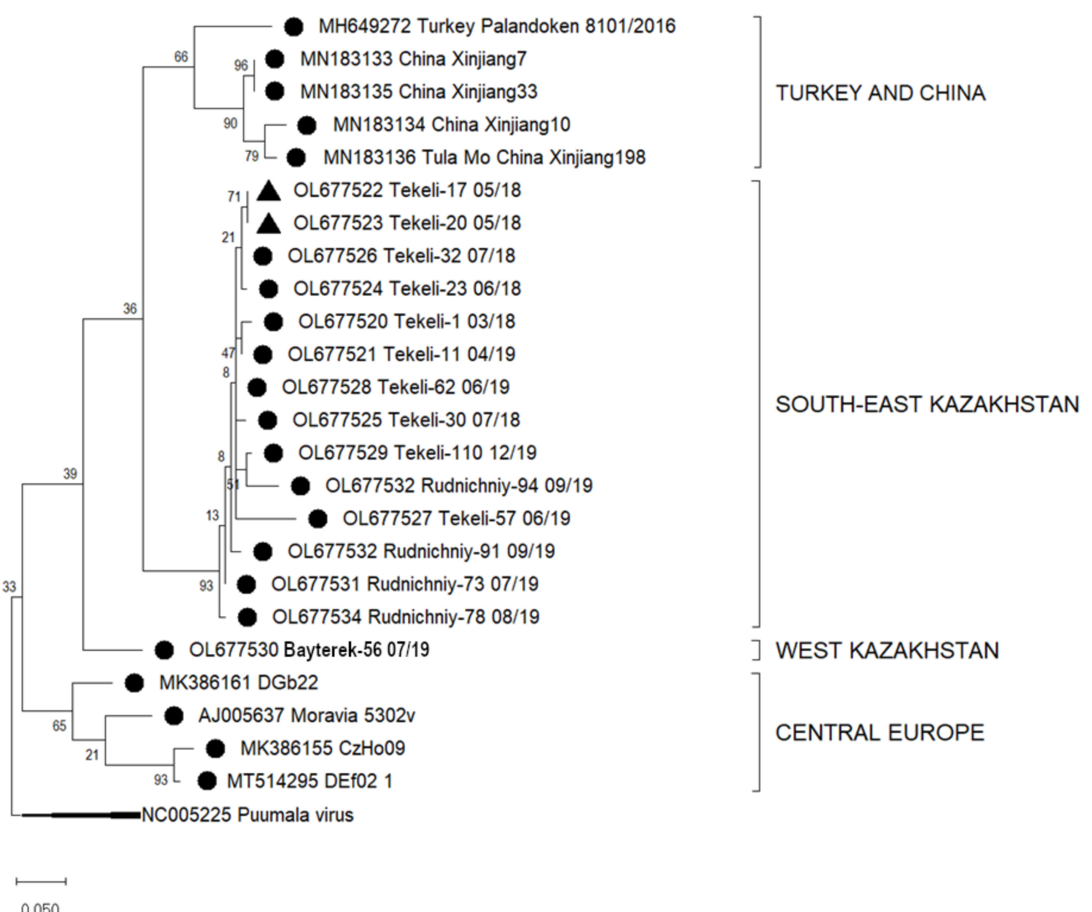

Figure 3. Phylogenetic analysis by Maximum Likelihood method of the L segments (184 nucleotides (nt), positions of sequences 5187–5371 nt in regard to the reference sequence NC005226) of Tula virus in Kazakhstan. The tree with the highest log likelihood (−1345.67) is shown. The percentage of trees in 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).

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 S1). 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 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**

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 geograph-

ically relevant regions reveals that the sequences have an identity range from 78.9–100% (Table 3).

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 south-east 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 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

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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Institutional Review Board Statement: Rodent trapping occurred 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 ethical committee of the Ludwig-Maximilian University in Munich, Germany (18-631).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used and/or analysed during the current study are available from the corresponding author on reasonable request. All determined sequences were uploaded to GenBank and are accessible as OL677520, OL677521, OL677522, OL677523, OL677524, OL677525, OL677526, OL677527, OL677528, OL677529, OL677530, OL677531, OL677532, OL677533, OL677534, ON239876, ON239877, ON239878, ON239879, ON239880, ON239881, ON239882, ON239883, ON239884, ON239885, ON239886, ON239887, ON239888, ON239889, ON239890, ON513437, ON513438, and ON513439.

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7 Discussion

The Republic of Kazakhstan is an emerging country. After the dissolution of the Soviet Union, the country managed to change from a minor developed state to a newly industrialized country. Nonetheless, in order to enable research on highly pathogenic agents, surveillance of natural endemic agents and development of new and modern diagnostics for patients, the state still needs supportive funding by third parties from other countries, such as the United States of America, France, the Netherlands and Germany (Peintner *et al.*, 2021).

In Kazakhstan there is a large number of not yet sufficiently studied potential natural foci for the vectors or reservoirs of some particularly contagious diseases, such as plague, anthrax, tularemia, brucellosis and many others. For example, the exposure of old animal burial sites by rain, earthquakes or settlement expansions can result in spontaneous outbreaks of anthrax.

Furthermore, Kazakhstan played a role in the research of biological warfare during the Soviet era. After the dissolution of the Soviet Union, all military facilities where proactively opened to the international community by the Republic of Kazakhstan and decontaminated in the 1990ies with international help. In 2004, Kazakhstan and the USA signed an agreement to start projects to further reduce the risk of biological weapons proliferation (Aikimbayev, 2016; Peintner *et al.*, 2021).

A literature search in the National Center for Biotechnology Information (NCBI) PubMed spanning the last 10 years revealed, however, how active research on a topic such as zoonosis and epidemiology is in certain countries. For Kazakhstan, there are only about 30 publications on zoonosis, which are available to the English-speaking science community. In comparison, more than 1600 publications on zoonosis are available from Germany in the same decade ("PubMed," 2022).

Countries, such as Germany are interested in what kind of natural endemic infectious diseases can be found in Kazakhstan and may pose a threat to Western countries. For this reason, the federal foreign office of Germany launched the German Biosecurity Programme. Its aim is to strengthen biosafety and biosecurity in partner countries based on the concept of the G7 Global Partnership against the proliferation of weapons and materials of mass destruction (Peintner *et al.*, 2021). Biosafety includes the safety of humans and the environment from the accidental release of pathogenic biohazards such as bacteria, viruses or toxins through the provision of policies and practices. In

contrast, biosecurity is the protection of humans and environment from an intentional release of biohazards by an individual by preventing access to and acquisition of knowledge, tools, and methods for their dissemination (Board on Life Sciences *et al.*, 2015). All studies conducted as part of this thesis were initiated and funded by this German initiative.

Epidemiological studies are a powerful tool to estimate the prevalence of specific pathogens in humans as well as in animals, which serve as host and also as vectors. Obtaining this information as part of the WHO´s One Health concept allows, for example, to carry out diagnostic measurements in an endemic area. In this study, material from patients, hosts such as rodents, and vectors such as ticks was examined for pathogens as shown in Figure 3 A-E.

Flaviviruses such as Tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and Omsk haemorrhagic fever virus (OHFV), members of genus *Orthohantavirus* and also bacterial agents such as *Rickettsia* spp. were the subject of the studies.

A main aim of these studies was to complete the information on these agents and to compare findings with previously published studies on these agents in Kazakhstan. In order to fully understand the spread of a zoonotic agent in a country, information must be gathered on the infection rate in humans, but also in their natural wild hosts and vectors, which are mostly ticks. The joint German-Kazakh cooperation started almost ten years ago. In those years some information has already been collected together with partner laboratories and biological institutes from Kazakhstan.

Viral pathogens, such as members of the family *Flaviridae*, that are also common agents to cause infectious diseases in Kazakhstan, have been part of this work. Among those, TBEV, which causes Tick-borne encephalitis (TBE), is endemic in North- and East Kazakhstan and Akmola and Almaty region (Figure 3 A) (SPC SEEM, 2021). A study has detected TBEV in ticks in official endemic regions, but also in non-endemic regions like Kyzylorda (Abdiyeva *et al.*, 2020). Approximately 30 to 40 cases of TBE are registered annually. In the last three years (2019-2022), a total of 90 cases of TBE infections have been registered in five of the 13 regions of Kazakhstan (Akmola, Almaty, East Kazakhstan, Zhambyl, North Kazakhstan). Three of the cases are known to have succumbed to the infection. Fatal cases are not recorded as consistently as infections, but from 2002 to 2021, 16 people have died from TBE infection, mainly in East Kazakhstan and Almaty region. Bevor 2019 TBE a few cases have also been recorded in Pavlodar and Kostnay region (Figure 3 A). As TBE can take different forms,

many cases remain undiagnosed. If the aetiological agent cannot be determined, patients are diagnosed with FUO or MUO. Up to 400 cases of MUO were recorded in Kazakhstan between 2017-2019 (SPC SEEM, 2021).
Part of this work was also a study, in which patients with suspected meningitis have been screened for potential causative agents like TBEV and WNV. (Shin *et al.*, 2022). In Kazakhstan, there is no official registration of WNV so far (Figure B). Information about WNV in this country is very limited. Studies show that WNV can be found in birds and mosquitos in West Kazakhstan region and that WNV specific antibodies are detectable in a few citizens of this region (Maikanov and Ayazbaev, 2016).
For this work, the human samples have been probed for TBEV and WNV by serological (TBEV and WNV IgM and IgG ELISA) and by indirect immunofluorescence assay (IIFA) (Yang *et al.*, 2019), and by molecular biological methods (real-time reverse transcriptase (RT) - PCR targeting the E-Gene of TBEV). The human samples (serum and CSF) were collected in three regions: Almaty, East Kazakhstan and Akmola. Out of 166 samples, only 31 samples from all three regions were positive for TBEV and WNV. It shows that for 80% of the investigated patients the infectious agent is still not known. Certain serological test systems (imported from Russia) are already available to detect TBE-infections in human patients in endemic regions in Kazakhstan, but also in non-endemic regions such as Almaty-, Akmola region and in East-Kazakhstan, where patient samples have been collected for this study. But as shown, still TBEV positive patients have been detected (Shin *et al.*, 2022). Doctors are not aware of a possible infection with TBEV or WNV, or the clinical symptoms are too mild, or clinical staff may recognize it, but no laboratory is nearby, which is familiar with TBEV or WNV serological test methods, to confirm the infection.

An additional member of the family of *Flaviviridae*, which can also cause severe infections in humans, is Omsk haemorrhagic fever virus (OHFV), which leads to the disease Omsk haemorrhagic fever (OHF) in humans. This viral zoonotic agent has not been detected in Kazakhstan before (Figure 3C), because the disease and its aetiological agent were only known to be present in Western Siberia in Russia (Kovalev *et al.*, 2021; Ruzek *et al.*, 2013). OHF outbreaks among the human population are reported in four regions of Russia (Kurgan, Tyumen, Omsk and Novosibirsk), bordering to North Kazakhstan.

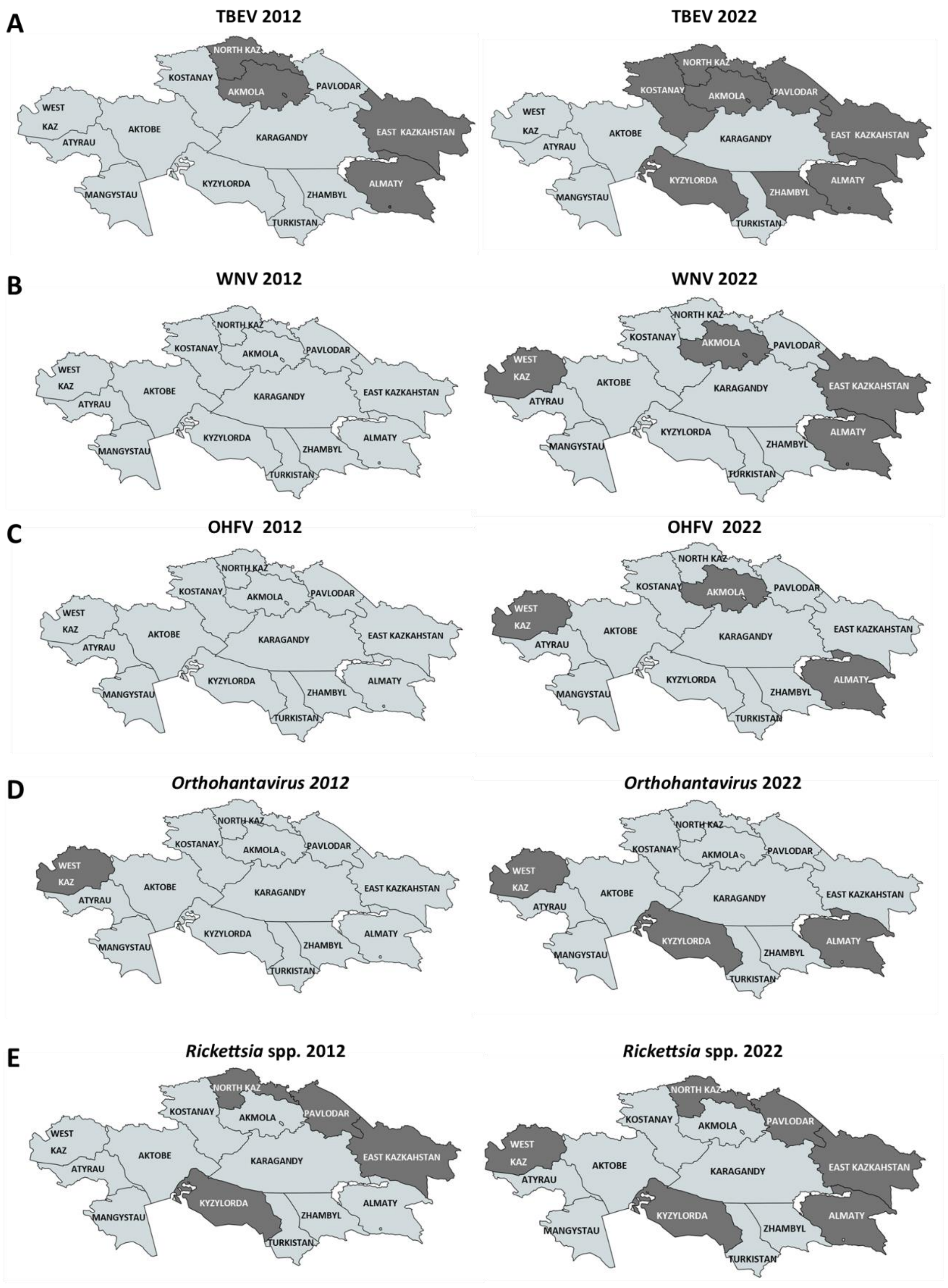

Figure 3 Comparison of the distribution of zoonotic agents and zoonosis in 2012 and in 2022, shown in A: Tick-borne encephalitis virus (TBEV); B: West Nile Virus (WNV); C: Omsk haemorrhagic fever virus (OHFV); D: *Orthohantavirus* and E: *Rickettsia* spp.. Some zoonotic infections are known to be prevalent in Kazakhstan in 2012, regions marked in dark grey are official endemic areas in 2012. After 10 years (2022) and several studies on different zoonotic diseases, additional, previously non-endemic areas, were identified and marked in dark grey. In 2022, regions marked in dark grey indicate areas that are officially endemic, but indicates also officially non-endemic regions, where the agents were detected in humans, hosts (small rodents and mammals) as well as vectors (ticks) according to latest reports and investigations. Maps generated with mapchart.net (“mapchart.net,” 2022).

Vectors such as *Dermacentor* or *Ixodes* ticks, the main mammal host *O. zibethicus*, and other OHFV associated rodent hosts like the vole *Arvicola* can also be found in Kazakhstan and can spread OHFV throughout the country. In our study, we analyzed human samples (CSF) and samples of potential hosts (rodents and mammals), as well as potential vectors (*Dermacentor* and *Ixodes* ticks). Our data showed for the first time that OHFV occurs also in Kazakhstan in human patients with suspected meningitis. Additionally, this virus has also been detected in their vectors (*Dermacentor* and *Ixodes* ticks), as well as in small mammals and rodents. These findings were confirmed by real-time-RT PCR positive samples derived from ticks from Akmola region, from rodents from West Kazakhstan region and two acute patients from Almaty region (Figure 3C) (Wagner *et al.*, 2022). These findings are important: due to the lack of knowledge and of diagnostic methods to indicate OHFV, Omsk haemorrhagic fever infections cannot be diagnosed.

Members of the genus O*rthohantavirus,* part of the family *Bunyavirales*, have also been examined as part of this work. *Orthohantavirus* causes haemorrhagic fever with renal syndrome (HFRS) and was first recorded in West Kazakhstan in 2000 (Figure 3 D). Since then, this area has been considered an endemic region for HFRS (Grazhdanov *et al.*, 2001). In West Kazakhstan, doctors reported severe forms of HFRS with haemorrhagic manifestations to the authorities. From 2000-2018, 245 cases of HFRS were registered and confirmed by serological methods (Bekmukhambetov, 2012; Zakharov *et al.*, 2010). In addition, longitudinal studies were performed to investigate the prevalence of *Orthohantavirus* in small rodents all over West Kazakhstan (Grazhdanov *et al.*, 2014). A study with antigen-testing has already been conducted in an uninhabited mountain area in the so far non-endemic Almaty region (Plyusnina *et al.*, 2008). This study revealed that some small mammals were positive for an infection with TULV. Thus, TULV only causes mild to nonclinical forms of HFRS. However, there are no officially registered cases in Almaty region so far. Nevertheless, a previously published study on FUO has shown that specific antibodies against *Orthohantavirus* are indeed present in serum samples from patients in Almaty and also in Kyzylorda region (Tukhanova *et al.*, 2020). In this work, West Kazakhstan and in the region of Almaty, small mammals have been analysed for the presence of *Orthohantavirus*. Furthermore, phylogenetic analyses have been performed by sequencing partial fragments of the small- and the large segment to support surveillance of circulating *Orthohantavirus* strains (Tukhanova *et al.*, 2022).

TULV was indeed found to be present in both regions (Figure 3 D) and phylogenetic analysis showed that analysed strains from West Kazakhstan are closely related to strains from Samara, Russia, located about 150 km from the trapping area of positive mammals in Uralsk. In the Almaty region, sequencing of TULV-positive samples from Tekeli and Rudniychy revealed a close relationship to another already published TULV strain found in Taldykorgan (distance 50 km). For the first time, TULV was detected in the forest dormouse (*Dryomus nitedula*), which is a new host for *Orthohantaviruses* (Tukhanova *et al.*, 2022).

Rickettsioses are a major challenge to the health system in Kazakhstan. Since 1995, more than 4,000 people have been infected with *Rickettsia* spp*.* but these cases are only recorded in endemic regions such as Pavlodar, Kyzylorda, North- and East Kazakhstan (Figure 3 E) (SPC SEEM, 2021). Especially in non-endemic areas, the diseases are often misdiagnosed. Mild forms are declared as FUO, but severe cases may be misdiagnosed as well. For example, two patients living in a CCHF endemic region (Kyzylorda) were misdiagnosed with CCHF upon admission to hospital. Not surprisingly, treatment with Ribavirin failed to cure the patients and ultimately both patients died. After their deaths, serological tests confirmed a *Rickettsia* infection (SPC SEEM, 2021). A recently published study on patients with FUO was also able to demonstrate that in a previously non-endemic region of Almaty, patients with *Rickettsia* specific antibodies were detected (Turebekov *et al.*, 2021).

Another study on ticks, which serve as vectors for *Rickettsia*, showed the occurrence of several *Rickettsia* species like *R. raoultii*, *R. slovaca* and a new species *R. yenbekshikazahkensis* in previously known endemic regions (Kyzylorda), but also in Almaty – so far declared as non-endemic region (Turebekov *et al.*, 2019).

As a consequence, the prevalence of *Rickettsia* spp. in their rodent hosts has been studied in two regions (West Kazakhstan and Almaty), where human Rickettsioses have not been clinically recorded and where no studies on their vectors (ticks) have been conducted so far. The new study presented in this work, showed and verified by sequencing that *R. conorii* is present in West Kazakhstan and *R. raoulti* and *R. slovaca* are present in rodents in Almaty region and that small rodents may also play a role in the life cycle of *Rickettsia* (Figure 3 E) (Wagner, E. *et al.* in preparation, for further information see supplemental). The role of rodents and small mammals in the *Rickettsia* life cycle is not clear yet (Brown and Macaluso, 2016; Burri *et al.*, 2014; Schex *et al.*, 2011; Tadin *et al.*, 2015). In this study, pinna have been analysed, which

seem to be a preferred spot for ticks and fleas on small mammals and rodents (Schex *et al.*, 2011). The genus *Rickettsia* consists of many *Rickettsia* species and it is also possible to detect more than one species in one infected host (Essbauer *et al.*, 2018; Turebekov *et al.*, 2019). For a clear and reliable differentiation, it is important to perform multilocus sequencing typing (MLST). Part of this method has also been applied in this work. After screening all DNA samples by real-time PCR targeting the pan-rickettsial citrate synthase gene (*gltA*), all positive samples were specified by targeting a partial outer membrane protein (*OmpAIV*) and the *23S-5S* interspacer region by conventional PCR. Sequencing results showed that *R. conorii*, *R. slovaca* and *R. raouliti* are circulating in small vertebrates in Kazakhstan, as they are also circulating in ectoparasites such as ticks and fleas all over Kazakhstan (Hay *et al.*, 2016; Kyraubayev *et al.*, 2014; Rudakov *et al.*, 2003; Sansyzbayev *et al.*, 2017; Shpynov *et al.*, 2005, 2004, 2001; Yegemberdiyeva and Shapieva, 2008). *Rickettsia* is widely distributed in hosts such as rodents and ticks and Rickettsioses are not reliably monitored or misdiagnosed in all areas of this country.

Kazakhstan has many zoonotic diseases and zoonotic agents. Studies for this work showed that viral agents such as OHFV, TBEV, WNV and members of the genus *Orthohantavirus*, as well as bacterial agents such as *Rickettsia* are more widely distributed in humans, small rodents and ticks as previously assumed (Figure 3). Based on these studies, the following questions may arise: Why and also how has the spread of different zoonotic diseases increased in this state?

The first and probably most plausible explanation is that the pathogens and diseases have always been distributed throughout the country. However, due to the lack of money and effort, it was not possible for Kazakhstan to establish more laboratories with capacities for examining BSL-3 and BSL-4 agents. Moreover, well trained personnel staff is missing and there is not enough funding to conduct studies all over the country. The agreement signed in 2004 between Kazakhstan and the USA was an addendum to the Nunn-Lugar Cooperative Threat Reduction to avoid the proliferation of biological weapons, technology, pathogens and expertise.

After signing the agreement, a new BSL-3 laboratory complex was built by the USA in Almaty. This so-called Central Reference Laboratory (CRL) now functions as the central laboratory for examining infectious diseases. Additionally, laboratories that served as biological weapon production facilities in former Soviet times are now laboratories with higher biosafety standards and modern technology to produce own

national vaccinations. One example is the Research Institute for Biological Safety Problems (RIBSP) based in Otar. In Nur-Sultan, the capital city of Kazakhstan, a modern laboratory can be found in the NCBI, where state-of-the-art sequencing is performed. Nevertheless, Kazakhstan needs several smaller laboratories throughout the country. In addition, the big laboratories struggle to find qualified personnel to work in these laboratories and there is still a lack of funding for the investigation of infectious diseases (Yeh *et al.*, 2021). Kazakhstan has come a long way since the dissolution of the Soviet Union and by its willingness to participate in international projects, it has already greatly improved the biosafety and biosecurity in its country.

Zoonoses are depend on the distribution of their hosts and vectors. One factor that should not be underestimated is the spread of hosts and vectors due to climate change or the transport of goods. Due to (altered) bird migration routes and the transport of goods such as grain and wheat by train or truck, rodents can easily travel hundreds and thousands of kilometres.

Additionally, Kazakhs tend to have strong family ties. Most Kazakh citizens live in or close to big cities such as Almaty, Nur Sultan or Shymkent. In order to travel to their summer quartiers (dachas) or to visit relatives, they often have to travel to more rural areas. Traveling, however, is a risk for exposure to ticks and small mammals. Furthermore, hunting and trapping of animals are favourite free time activities in these rural areas. Sanitary and electrical facilities are also very basic in rural regions. Consumption of food that is not well cooked or milk that is not pasteurized is also common. All of these factors may lead to the spread of infections and zoonotic diseases.

From these suspected causes for the increasing prevalence of zoonotic diseases, some recommendations for Kazakh policy can be derived. Efficient epidemiological studies and biosurveillance are very costly. However, there is an urgent need to provide more money for scientific studies on dangerous infectious diseases and to establish modern laboratories in the existing network of Anti-Plague Stations with adequate personal protection equipment (PPE) for working with BSL-3 agents. Once the infrastructure is in place, studies have to be conducted regularly in all regions. The results of these studies have to be reported transparently, including to the international scientific community in English. *Vice versa*, it would also be important to disseminate English literature in Russian language, in order to provide more knowledge and information to central Asian countries. In addition, clinical personnel and physicians

have to be trained and sensitized for infectious diseases, especially for those that are not endemic in certain regions. Studies show that *e.g. Rickettsia*, TULV, and OHFV can be found in hosts such as rodents and also in vectors such as ticks all over Kazakhstan, regardless of whether areas are officially endemic or not (Abdiyeva *et al.*, 2020; Turebekov *et al.*, 2019; Wagner *et al.*, 2022). These pathogens such as CCHF, TBEV, WNV, *Orthohantavirus* and OHFV cannot only be found in wild living animals but also in humans in non-endemic regions in Kazakhstan (Abdiyeva *et al.*, 2019; Shin *et al.*, 2022; Tukhanova *et al.*, 2019; Wagner *et al.*, 2022). Our study of patients with meningitis of unknown origin (MUO) represents as a good example. Of 166 tested patients with suspected meningitis, 31 showed reactivity for TBEV and WNV (Shin *et al.*, 2022). In two patients OHFV was detected using real-time-PCR (Wagner *et al.*, 2022). In total, for approximately 20% of the patients the potential causative agent was determined – for 80% of patients with meningitis, however, the causative agent is not known. Since these results also include the detection of IgG, which might reports an old infection, the actual number of undetected aetiologies is even higher.
The diagnostic tools used in this study were more or less new to scientists and doctors in Kazakhstan. First, specific PCR protocols had to be established (Peintner *et al.*, 2021). In addition, there is a lack of laboratories and trained personnel to carry out reliable serological tests. Additionally, physicians often were not aware of and not sensitized to any of these infectious diseases. Since cross-reactivity plays a crucial role in the evaluation of serological tests, it would be necessary to send samples to other laboratories for a second examination (Colomba *et al.*, 2006; Rathore and St. John, 2020), in order to verify the results. Furthermore, samples have to be sent to international reference laboratories for specific infectious diseases as internal control mechanism. In addition, if Kazakhstan would participate in international interlaboratory tests according to international standards, the quality of scientific approach would be increased enormously. Unfortunately, Kazakh law does not allow the import and export of biological samples.
In this work, highly relevant zoonotic pathogens in Kazakhstan were examined. The establishment of the studies and methods in this country and the reported results lay the foundation to foster biosecurity and biosafety in Kazakhstan, which is home to many life-threatening pathogens due to its enormous size and the huge number of different landscapes.

8 Concluding remarks

The implementation of new diagnostic methods in emerging countries enables an improvement in the quality of patient care and epidemiological surveillance. The central Asian country of Kazakhstan has a diverse fauna, with many particularly dangerous endemic pathogens leading to infections such as like CCHF, TBEV, *Orthohantavirus*, or *Rickettsia* (Peintner *et al.*, 2021). This work has focused on the investigation of those pathogens in humans, hosts (rodents) and vectors (ticks) in non-endemic regions in Kazakhstan.

In a study with 166 patients with suspected meningitis, the aetiology of the agent could be determined in only 33 patients. 31 of them were diagnosed with TBEV or WNV and were examined with serological and molecular methods, which were new to Kazakhstan. Due to the high cross reactivity among *Flaviviruses*, ELISA and IIFA methods were compared, and, if possible, a molecular biological confirmation of the virus species identified was carried out using a specific PCR. The results show that ten cases of meningitis of unknown origin from different regions in Kazakhstan were caused by TBEV or WNV (Shin *et al.*, 2022).

Furthermore, in two of the samples of CSF collected from patients with suspected meningitis, OHFV was also detected using molecular biological methods. This indicates acute cases of patients suffering from OHFV. Previously, OHFV was known to be prevalent only in four regions of Russia bordering northern Kazakhstan (Shin *et al.*, 2022; Wagner *et al.*, 2022).

An epidemiological screen for OHFV in potential hosts such as small mammals and vectors (*Dermacentor* and *Ixodes ticks)* revealed that OHFV may be more widespread throughout Kazakhstan than previously assumed (Wagner *et al.*, 2022).

Another widespread virus in Kazakhstan is *Orthohantavirus*. An analysis of rodent lungs revealed that *Orthohantavirus*, such as the species TULV, is widespread throughout Kazakhstan and can also be found in currently non-endemic regions of Kazakhstan in rodents like the common vole (*Microtus arvalis)*, but also in the dormouse *Dryomys nitedula*. This species was not known as a potential host before. A phylogenetic analysis revealed distinct geographic clusters of the genome sequences isolated from the viruses. Further studies of *Orthohantavirus* in rodents

would be necessary to determine if there are more geographic clusters or how widespread each cluster is in Kazakhstan (Tukhanova *et al.*, 2022).

Rodents also serve as hosts for members of the genus *Rickettsia*. Analysis of pinnae collected from small rodents revealed several infected animals. A subsequent verification by sequencing showed that the species *R. conorrii*, *R. raoulti* and *R. slovaca* can be detected in rodents. Even though it was known that *Rickettsia* also exists in ticks and infected humans in this area, this is the first description of *Rickettsia* in their long term host (Wagner, E. *et al.* in preparation, for further information see supplemental).

These studies highlight that Kazakhstan has a large, still unexplored reservoir of pathogens. In order to gain more information on potential threats for the public health in Kazakhstan, further studies on other zoonotic pathogens and their natural habitat in this country would be necessary.

To implement these studies, however, further national and international funding is needed in order to sustainably build and strengthen laboratory capabilities call over Kazakhstan. In the long run, this will improve the treatment of infected patients or prevent infections with extremely dangerous pathogens in the first place.

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Appendix

Unpublished work

Incidence of *Rickettsia* species in rodents in two regions in Kazakhstan

Edith Wagner, Anna Shin, Nur Tukhanvoa, Nurkeldi Turebekov, Zhanna Shapiyeva, Alexander Shevtsov, Talgat Nurmakhanov, Vitaliy Sutyagein, Almas Berdibekov, Nurbek Maikanov, Ilmars Lendyzish, Klaus Freimüller, Rosina Ehmann, Christina Ehrhardt, Sandra Essbauer and Lukas Peintner.

RESEARCH PAPER

Incidence of *Rickettsia* species in rodents in two regions in Kazakhstan

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Keywords: *Rickettsia* spp., rodents, small mammals, Republic of Kazakhstan

Abstract:

Records on the distribution of *Rickettsia* spp. in their natural hosts in Central Asia are incomplete. The first description of Rickettsioses was made in the 1950ies in the city of Almaty in the Republic of Kazakhstan. Currently, endemic areas are North Kazakhstan, Pavlodar, East Kazakhstan and Kyzylorda. However, a recent study reported the presence of murine/endemic typhus in Almaty and Kyzylorda regions in arthropod vectors. Wild animals such as rodents can act as natural reservoirs for *Rickettsia* spp. to maintain a beneficial lifecycle. Such data on the natural lifecycle of *Rickettsia* are available for Western countries, but - to our knowledge - no data are available in Kazakhstan so far. Here we present for the first time data on *Rickettsia* spp. detected from rodent ears (n=624) in two regions in Kazakhstan. *Rickettsia* spp. DNA was extracted from ear pinna and screened by real-time PCR targeting the pan-rickettsial citrate synthase gene (*gltA*). From all analysed small mammals 17 (2.72%) were positive for *Rickettsia* spp. A subsequent sequencing of the rickettsial gene *OmpAIV* and *23-5S* interspacer region revealed the same heritage of identified rickettsia species as observed in ticks of previous studies from the region. In summary, this study proved that rodents in Kazakhstan serve as a natural reservoir of *Rickettsia* spp.

Introduction:

Rickettsiae are small (0.3 to 0.5 by 0.8 to 2.0 μm) gram-negative intracellular bacteria, living in the cytosol of their hosts (1, 2). The genus *Rickettsia* is divided into four groups. (i) The spotted fever group (SFG) linked predominantly with ticks and less often with fleas and mites, including Mediterranean-, Rocky Mountain- and Helvetica spotted fever and Queensland tick typhus, with more than 30 species. (ii) The typhus group (TG) which includes agents of epidemic typhus and murine typhus associated with lice and fleas. (iii) An ancestral group with *R. bellii* and *R. canadensis* and (iv) a transitional group with members of *R. akari* and *R. felis*.

The SFG, TG, and transitional groups include agents qualified to cause disease in human (1, 3). SFG is distributed worldwide and includes more than 30 species. At least 15 species cause disease such as *R. rickettsii* in North America leads to Rocky Mountain spotted fever (RMSF) or *R. conorii,* causing Mediterranean spotted fever (MSF) in parts of Europe, Africa, and Asia (4–9). From the typhus group, *R. typhi* is causing murine/endemic typhus and more seldom *R. prowazekii* is inducing louse-borne or epidemic typhus in humans (4, 10, 11). Transitional group comprises of three species transmitted by different vectors that are *R. akari* transmitted by mites (rickettsialpox), *R. australis* transmitted by ticks (Queensland tick typhus), and *R. felis* transmitted by fleas (flea-borne spotted fever) (9, 12–17).

Rickettsioses are generally distributed worldwide (4). Sparse information is available on the disease and the distribution of tick-transmitted infections like Rickettsioses in Asian countries, but it is known that SFG and TG Rickettsia are present in Southeast Asia (4, 18–21). However, there are only incomplete records on the distribution of *Rickettsia* in the Central Asia. In a representative country for the region, the Republic of Kazakhstan, most available information is based on anecdotal reports as described during an expedition by Bartoshevic to the region of Almaty in 1949-1951 (22). In 1965 clinical pictures of tick-borne rickettsioses were observed in South Kazakhstan, West Kazakhstan, Pavlodar, North Kazakhstan and Akmola region (23). In 1961, *R. sibirica* was detected in *Dermacentor marginatus* and *Haemaphysalis punctata* ticks collected from the Yenbekhikazakh district in Almaty region (24). Other studies confirmed, that *R. conorrii* ssp. *caspia, R. slovaca, R. raoultii,* and *R. felis* are circulating throughout Kazakhstan (25–32).

Official endemic regions in Kazakhstan are currently North Kazakhstan, Pavlodar, East Kazakhstan and Kyzylorda. From 1995 to 2021 a total of 4,627 human cases of tick-borne rickettsioses were reported in Kazakhstan. In the past years the incidents rates in Kazakhstan are increasing starting from 1995 with 0.41 (per 100,000 inhabitants per year) with the highest number of rates from 1.19 in 2018 and 1.12 in 2019. The biggest increase from 1995 to 2021 was observed in 2019 in Kyzylorda region (incidence value of 1.64 - 12.68) and in Pavlodar region (incidence of 1.07 - 9.15) (33).

While Rickettsioses are monitored and reported in patients in Kazakhstan relatively little is known about the spread of this zoonosis in the fauna of Kazakhstan. A recent study on the prevalence of rickettsia species in ticks in Almaty and Kyzylorda regions revealed a minimum infection rate (MIR) of 0.4-15.1% in Almaty region and 12.6-22.7% in Kyzylorda region. The detected species were *R. raoultii*, *R. slovaca,* a new *Candidatus R. yenbekshikazakhensis*, and the new genotype of *R. talgarensis* (34).

Wild animals act as a natural reservoir for *Rickettsia* spp. and maintain their life cycle in nature (35, 36). Some data on the natural life cycle of *Rickettsia* spp. are available from Europe, but no data from Central Asia are published so far. The European studies showed that screening ear pinnae of small mammals is an adequate tissue to detect *Rickettsia* species (37).

Here we aim to detect *Rickettsia* spp. in ear pinnae of small mammals in West-Kazakhstan and Almaty region to understand the distribution and the heritage of *Rickettsia* spp. in both regions.

Material and Methods:

Collection of tissues from small mammals

Rodent trapping was conducted upon ethical approval of Kazakhstan local ethics committee at the National Scientific Center for Especially Dangerous Infectious in Almaty, Kazakhstan (protocol #4, 08.01.18) and the ethical committee of the Ludwig-Maximilians-University in Munich, Germany (opinion number 18-631) with snap traps in 2018 and 2019. In West-Kazakhstan region, small mammals were trapped in 19 trapping sites of the four districts Bayterek, Borili, Terekti, and Taskala. In Almaty region, small mammals were trapped in the three districts Tekeli, Rudnichniy, and Bakanas. In Almaty city small mammals were trapped in seven trapping sites (detailed location information see Supplementary Table 1 and (38). From the 624 trapped small mammals, ear pinnae were removed aseptically and stored in RNALater (ThermoFisher Scientific, Waltham, United States), at -20°C.

DNA extraction

Ear pinnae from small mammals were and homogenized with two stainless steel beads and 1 ml of cell culture medium (Gibco™ MEM, ThermoFisher Scientific, Massachusetts, United States) using the TissueLyser II (2 min at 30 Hz) (Qiagen, Hilden, Germany). The homogenized samples were centrifuged for 5 min at 2,348 g. DNA was extracted from 350 µl of the supernatant using QiAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -20°.

Real-time PCR approach

A real time PCR assay to screen for rickettsial DNA in the rodent ear pinnae was performed using the LightCyclerFastStart DNA Master HybProbe System (Roche) for RotorgeneQ (Qiagen, Hilden, Germany) targeting the pan-rickettsial citrate synthase gene (*gltA*). An Uracil-DNA-glycosylase (UDG) incubation step was added to get rid of any carry-over PCR products between the reactions (37, 39). Employed primers were PanRick_*gltA*_2 forward (5′-ATA GGA CAA CCG TTT ATT T-3′), PanRick_*gltA*_2 reverse (5′-CAA ACA TCA TAT GCA GAA A-3′), and the probe PanRick_*gltA*_2_taq (5′-6FAM-CCT GAT AAT TCG TTA GAT TTT ACC G-DB-3′) (34, 37, 39).

Conventional PCR to generate DNA fragments for sequencing

Real-time PCR positive samples were further investigated with a conventional PCR to amplify a fragment of the *outer membrane protein OmpAIV* (primers RR 190-5125: 5'-GCG GTT ACT TTA GCC AAA GG-3', cRR 190-6013: 5'-TCT TCT GCG TTG CAT TAC CG-3')(37, 40, 41) and the *23S-5S* interspacer region (23s forward: 5'-GAT AGG TCG GGT GTG GAA GCA C-3', 23s reverse: 5'-GGG ATG GGA TCG TGT GTT TCA C-3')(42) according to published protocols (34, 37, 40–42) for subsequent sequencing. PCR products were analysed on agarose gels expecting a band at 378-532 bp for the *23S-5S interspacer* region (42) and 888 bp for the *OmpAIV* (41).

Sequencing

All conventional PCR products targeting the partial *OmpAIV* and *23S-5S* interspacer region were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing was performed according to manufacturer's instructions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) and a 3730xl DNA Analyzer (Applied Biosystems, Japan).

Phylogenetic Analysis

Before BLAST-aided species determination and phylogenetic tree analysis the primer sequences were deleted from the sequences and then aligned in Bioedit 7.2.5 (43). Nucleotide sequence analyses were performed with Chromas Lite, version 2.1 (Technelysium Pty Ltd, South Brisbane, Australia) and compared for similarity to sequences deposited in GenBank. Phylogenetic trees were constructed in MEGA X with the Maximum Likelihood method based on the Tamura 3-parameter model (44). Obtained *OmpAIV* and 23S-5S IGS nucleotide sequences were deposited in GenBank database under accession numbers ON604636, ON604637, ON604638, ON604639, ON604640, ON604641, ON604642, ON604643, ON604644, ON604645, ON604646, ON604647, ON604648, ON604649 and ON604650.

Results:

Eleven species of small mammals were collected at 29 trapping sites from West-Kazakhstan region, Almaty region and Almaty city in the years 2018 and 2019 (Figure 1). This 624 investigated small mammals can be grouped in rodents and insectivores. Members of the families Cricetidae (*Microtus arvalis* (n=87), *C. glareolus* (n=13) and *Microtus kirgisorum* (n=49)), of Muridae (*Apodemus uralensis (n=259), Mus musculus (n=128), Rattus norvegicus (n=39), Meriones meridianus (n=2)*) and of Gliridae (*Dryomys nitedula* (n=15)) were examined. In addition, insectivores like *Crocidura suaveolens* (n=28) and members of *Sorex* spp. (n=4) were part of the study (Supplemental table 1).

Out of all the 624 screened ear pinnae of the collected small mammals 17 (2.72%) responded positive for the pan-*Rickettsia* citrate synthase gene *gltA* (Table 1). They were detected in rodents captured either in Bakanas (Almaty region) in *M. musculus* (n=8) and in *M. meridianus* (n=1) or in the area of Tekeli in *M. arvalis* (n=2) and *A. uralensis* (n=1), 200 km to the east of Bakanas. In addition, in West-Kazakhstan region in total five positive samples were detected in Bayterek region with a prevalence of 2.3% (n=5/220) in *A. uralensis (n=4)* and *M. arvalis* (n=1).

The prevalence for a rickettsia infection in the species itself is varying for the region. In Almaty region the prevalence is 50% for *M. meridianus* (n=1/2), 12% for *M. musculus* (n=8/66), 2.7% for *M. arvalis* (n=2/74), and 0.76% for *A. uralensis* (n=1/131), whereas in West-Kazakhstan region the prevalence of rickettsial DNA is 3.13% in *A. uralensis* (n=4/128) and 7.7% in *M. arvalis* (n=1/13).

Of all 17 *gltA* real-time PCR positive rodents, conventional PCR for detecting a part of the *outer membrane protein OmpAIV* and of the *23S-5S interspacer* region was performed to gain more information about the species of detected the rickettsia. In total 18 sequences were obtained, nine partial *OmpAIV-,* and nine partial *23S-5S interspacer* region sequences. The partial *OmpAIV* sequences, all obtained in 2018, are from the villages Tekeli (n=1, AO-Tek-2018-34) and Bakanas (n=6, AO-Bak-2018-1,-2,-3,-5,-7 and -13) in Almaty region and from the Bayterek area (WKO-Bay-2018-26 and -39) in West Kazakhstan region.

The partial *23S-5S* fragments are from Almaty region Bakanas (AO-Bak-2018-1,-2,-3,-5,-7,-8,-13) and WKO Bayterek region (WKO-Bay-2018-20), all obtained in 2018 and one from 2019 (WKO-Bay-2019-40). All attained sequences were compared to publicly

available sequences deposited in the GenBank database using NCBI BLAST and *R. raoultii*, *R. slovaca*, or *R. conorii* returned as the putative species detected in the ear lobes.

In six samples both *OmpAIV* and *23S-5S* sequences were obtained from the same ear lobe (AO-Bak-2018-1,-2,-3,-5,-7 and -13). However, only four of them yielded long enough sequence reads for a reliable phylogenetic analysis. Two too short 23S-5S sequences (AO-Bak-2018-5 and -7) had to be excluded from the pedigree exploration (Table 1, marked with ***).

Three of the four paired samples showed in both obtained gene loci (*OmpAIV* and *23S-5S)* species *R. raoultii (*AO-Bak-2018-1,-2 and -3). However, one of the paired samples (AO-Bak-2018-13) showed different species affiliations for *OmpAIV* and *23S-5S*. In the *23S-5S* fragment it revealed high sequence similarity (99.74% identity) to *R. raoultii* (strain Khabarovsk CP010969) but the partial *OmpAIV* sequence clustered with a very high resemblance (100% identity) to a published *R. slovaca* sequence from Tekeli (MG973999) (34).

This ambiguity of the species can also be observed in the phylogenetic trees in Figure 2 and 3, where representatives of worldwide distributed *Rickettsia* species and also published *Rickettsia* sequences from Kazakhstan like *R. raoultii* from Tekeli and Kyzylorda as well as recently recorded "*Candidatus Rickettsia yenbekshikazkahensis*" and "*genotype rickettsia talgarensis"* (34) are included. AO-Bak-2018-13 clusters in Figure 2 for *OmpAIV* with other strains of *R. slovaca* (MG973999 and CP002428) and in Figure 3 for 23S-5S with representatives of *R. raoultii* from Tekeli (MG974041 and MG974047).

For some *gltA* positive ear lobes only one sequence, either from *OmpAIV* or *23S-5S* could have been generated. Three partial sequences for *OmpAIV* (AO-Tek-2018-34, WKO-Bay-2018-26, and WKO-Bay-2018-39) showed high similarity to *R. raoultii* (Tabele 1). In addition, three individual sequences for 23S-5S (AO-Bak-2018-8, WKO-Bay-2018-20, WKO-Bay-2019-40) clustered with *R. raoultii, R. slovaca* or *R. conorii*, respectively (Figure 2 and Table 3).

Discussion:

To our knowledge, this study shows the first large-scale investigation of *Rickettsia* of in rodents in Kazakhstan. It is a follow up study to recently published investigations on *Rickettsia* in ticks (34) as well as agents for fever of unknown origin in patients (45). Hence, it closes the link from vectors and the disease in humans since it investigates the prevalence in natural hosts. Two regions of Kazakhstan were part of the study, the West-Kazakhstan region and the Almaty region in the south-east of the country including Almaty city. Both regions are not yet listed as endemic areas of rickettsiosis in Kazakhstan. Currently official endemic areas for rickettsioses in Kazakhstan are North-Kazakhstan, Pavlodar, East-Kazakhstan and Kyzylorda (Figure 1). Only in these endemic regions the numbers of infections and incidences are recorded and listed in annual reports on case numbers (45, 46). However, a previous study in Kazakhstan on fever patients enrolled in Kyzylorda, an endemic region, and Almaty region, a non-endemic region, showed that in both regions 1.4% of 802 patients had acute SFG rickettsioses and 2.7% acute TG rickettsioses. A previous infection with SFG or TG rickettsia was detected in approximately 30% of the participating patients (45). This study on patients was backed by a further investigation in ticks collected in the same regions (Almaty and Kyzylorda). Here, several rickettsia species were identified in the arthropod vectors (34). The minimum infection rate (MIR) for rickettsia in the investigated ticks (*Dermacentor marginatus, D. reticulatus, Haemaphysalis punctata, Hyalomma asiaticum, and Rhipicephalus turanicus*) in Kyzylorda region was 12.6-22.7%, and in the non-endemic Almaty region 0.4-15.1%. In those ticks the species *R. raoultii* and *R. slovaca*, a new form “*Candidatus R. yenbekshikazakhensis*” and a new genotype “*genotype R. talgarensis*” was detected (47). The role of vectors has also been assessed in further studies. For instance, several *Rickettsia* species were detected in ticks and fleas collected all over Kazakhstan including Kyzylorda, East Kazakhstan, West-Kazakhstan and Almaty region) (25–28, 30, 31). At the Kazakhstan-China border in the Chinese province of Xinjiang several Rickettsia species (*R. raoultii, candidatus R. barbariae* and genotype *Babesia*) were detected in *Haemaphysalis* ticks that were collected from *Vormela peregusna* (marbled polecats) (48). These publications showed, that rickettsia are more widely distributed in Kazakhstan as officially thought and also resides in non-endemic areas like Almaty region. Misclassification of endemic regions might lead to a problem for physicians to misdiagnose this disease in infected patients.

However, the role of rodents and small mammals in the life-cycle of rickettsia is not clarified yet (37, 49–51). Ticks may transmit *Rickettsia* transovarially and also transstadially, which empowers the spread of the bacteria within the tick population without any additional vertebrate reservoir (52). Still, an infection of vertebrates during the blood feast of ticks is highly probably. Indeed studies highlight that small animals - living in the wild or in laboratories - act as potential reservoir hosts for *Rickettsia* species (53–55). Other studies, however, claim that rodents and small mammals do not carry any rickettsial DNA (50, 56–59).

Here we show that rickettsia species can be detected in the ear pinnae of several families of small mammals such as *Cricetidae* (*M. arvalis*) and *Muridae* (*M. musculus* and *A. uralensis*). The ear lobes are a favourable spot for ectoparasites like ticks and fleas that are feeding on rodents and other small mammals (37). However, here we could not investigate if rodents with rickettsia infected ears would also yield a positive PCR result when screening alternative organs. Other studies showed that rickettsia DNA can be detected in blood and skin biopsies (like ear pinnae), however with stark differences (55, 60). It is reported that the amount of rickettsia DNA in skin biopsies is threefold compared to the rickettsia DNA content of blood, spleens have even lower titres in infected animals (60).

From 624 screened small mammals, 17 reacted positive in a *gltA* screening PCR. This is a surprisingly high number given the fact that a screen using PCR as the diagnostic tool only identifies animals that have an acute infection with rickettsia. The bacteraemia phase is in rodents rather short (61), however this is the time window where ticks might get infected while feeding. The rodents that yielded a positive *gltA* PCR such as *M. musculus* or *M. arvalis* are typical targets of *Dermacentor marginatus*, a tick reported to carry *R. raoultii* and *R. slovaca* in previous studies in the investigated areas (47).

To gain an idea on the genotypes circulating in small mammals in Kazakhstan we further amplified and sequenced two partial gene loci, *OmpAIV* and *23S-5S*, from the *gltA* positive samples. Of 17 positive ear pinnae we could gain six partial fragments of *23S-5S interspace* regions and nine partial *OmpAIV* fragments. By using BLAST it was possible to identify the potential genotype of the rickettsia infecting the rodents. All identified sequences had high similarities to either *R. raoultii*, *R. slovaca, or R. conorii*. All three have been reported previously to reside in ticks in Kazakhstan (63) and are of the SFG group. *R. slovaca* and *R. raoultii* are human pathogens that may cause

scalp eschar and neck lymph adenophaty after a tick bite (SENLAT) syndrome that was also reported in Kazakhstan (62).

A phylogenetic analysis of the obtained sequences with other sequences deposited in GenBank shows that the amplified fragments cluster closely with other rickettsia sequences that were isolated from ticks or small mammals in the region. All sequences isolated in the village of Bakanas had a close sequence relationship to sequences obtained from ticks isolated in Tekeli, a city from the same region in Kazakhstan. We are the first to sequence partial rickettsia genomes in the West Kazakhstan region, more than 2000 km to the west from the sampling sites in Almaty region. Still the phylogenetic distance is very short. This either proves that the genome of rickettsia is highly evolutionary conserved (62, 63), or allows the alternative explanation that rickettsia only recently moved to West Kazakhstan by migratory small mammals or ticks on birds.

Unfortunately, not from all positive *gltA* samples *OmpAIV* or *23S-5S* sequences could be obtained. Other studies already showed that conventional PCR assays are less sensitive than real-time PCR assays (55). This explains, why some lysates yielded positive results in the real time PCR but failed to produce a product in the conventional PCR that was conducted in order to gain longer fragments for phylogenetic analysis.

In summary this study highlights that rickettsia bacteria can be detected in small animals in non-endemic areas like Almaty region and West-Kazakhstan region. In this areas rickettsia infections are not monitored and a number of undifferentiated or misdiagnosed patients with rickettsiosis can be assumed, like postulated in a previous patient study in Almaty region (45). Hence, physicians and policy makers in the Republic of Kazakhstan should be aware that rickettsia diseases are more widespread than previously thought.

Conflict of Interest:

The authors declare no conflict of interest. The authors declare that there is no financial or personal relationship with other people or organisations that could inappropriately influence the work. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by Bundeswehr Joint Medical Service or any other governmental institutions.

Author contributions:

EW, LP and SE conceived the layout of the project. ASi, NTk, NTu performed homogenization and DNA-Extraction. ASi, NTk, EW performed experimental work, ASe was in charge of the sequencing, EW performed analysis of data, created figures and tables and wrote the manuscript. ASi, NTk, NTu, ZS, ASh, TN, VS, AB, NM, IL, KF, RE and CE contributed additional information and reviewed the manuscript. SE and LP supervised the project. LP was in charge of the revision process.

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Data Availability Statement:

The data used and/or analysed during the current study are available from the corresponding author on reasonable request. All generated sequences were uploaded to Genbank and are accessible as ON604636, ON604637, ON604638, ON604639, ON604640, ON604641, ON604642, ON604643, ON604644, ON604645, ON604646, ON604647, ON604648, ON604649 and ON604650.

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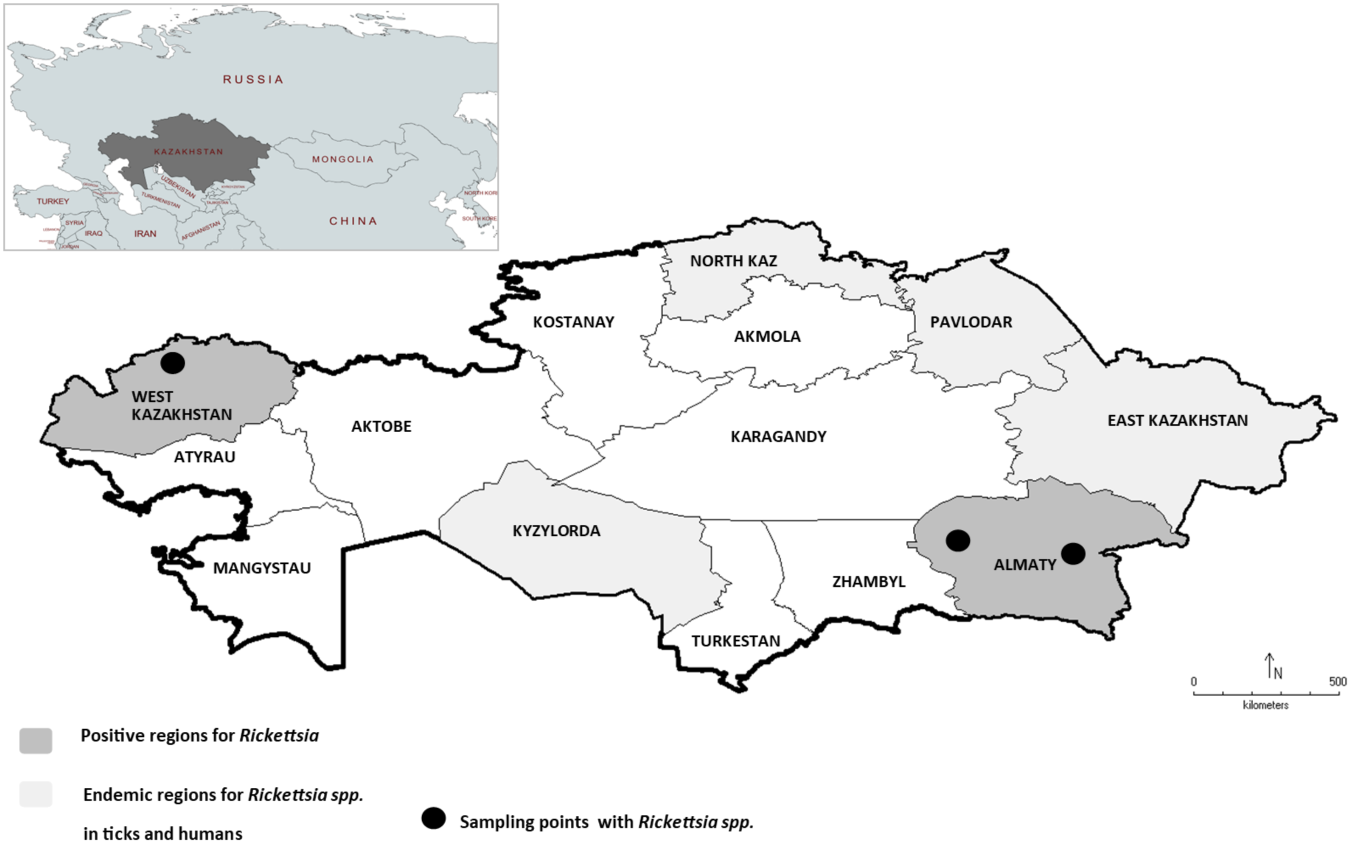

Figure 1: Investigation of *Rickettsia* spp. in Kazakhstan. Rickettsioses in humans are endemic in North Kazakhstan, Pavlodar, East Kazakhstan and Kyzylorda (light grey marked areas). Small mammals and rodents were investigated in West Kazakhstan and Almaty region (dark grey marked areas) with indicated positive sampling spots (● in 2018 and 2019. In West-Kazakhstan in the area of Bayterek and in Almaty region in Tekeli (left ● and Bakanas (right ●.

Table 1: Results of *Rickettsia* spp. investigation. 624 small mammals from Almaty and West Kazakhstan region sampled in 2018 and 2019 have been first screened by a real-time PCR targeting the *citrate synthase gene (gltA).* Positive rodents have been further investigated by conventional PCR targeting a fragment in the *outher membrane protein* (*OmpAIV*) and the *23S-5S interspacer* region. Obtained sequences were deposited at GenBank (ON604639-ON604650). All gained sequences have been identified by BLAST and were compared in the phylogenetic trees of Figure 2 and 3.

Region	Year	Sample ID	Rodent Species	*gltA* - citrate synthase (real time PCR)	*OmpAIV* - Outer membrane protein A: Sequence check by BLAST Rickettsia species per Ident [%]	Species according Fig.2	GenBank ID	*23S-5S* - Interspacer region: Sequence check by BLAST Rickettsia species per Ident [%]	Species according Fig. 3	GenBank ID
Almaty region	2018	AO-Tek-2018_32	*M. arvalis*	*positive*	X	X		X	X	
		AO-Tek-2018_34	*M. arvalis*	*positive*	*R. raoultii per Ident 99.8%*	*R. raoultii*	ON604636	X	X	
		AO-Bak-2018_1	*M. musculus*	*positive*	*R. raoultii Isolate Tekeli 041 100%*	*R. raoultii*	ON604637	*R. raoultii strain Khabarovsk 99,7%*	*R. raoultii*	ON604645
		AO-Bak-2018_2	*M. musculus*	*positive*	*R. raoultii strain Khabarovsk 100%*	*R. raoultii*	ON604638	*R. raoultii strain Khabarovsk 100%*	*R. raoultii*	ON604646
		AO-Bak-2018_3	*M. musculus*	*positive*	*R. raoultii Isolate Tekeli 041 100%*	*R. raoultii*	ON604639	*R. raoultii strain Khabarovsk 99,7%*	*R. raoultii*	ON604647
		AO-Bak-2018_5	*M. musculus*	*positive*	*R. raoultii strain Khabarovsk 100%*	*R. raoultii*	ON604640	*R. raoultii strain Khabarovsk 100%*	***	
		AO-Bak-2018_6	*M. musculus*	*positive*	X	X		X	X	
		AO-Bak-2018_7	*M. musculus*	*positive*	*R. raoultii strain Khabarovsk 99,9%*	*R. raoultii*	ON604641	*R. raoultii strain Khabarovsk 99,6%*	***	
		AO-Bak-2018_8	*M. musculus*	*positive*	X	X		*R. raoultii strain Khabarovsk 100%*	***	
		AO-Bak-2018_13	*M. musculus*	*positive*	*R. slovaca isolate Tekeli 100%*	*R. slovaca*	ON604642	*R. raoultii strain Khabarovsk 99,74%*	*R. raoultii*	ON604648
		AO-Bak-2018_14	*M. musculus*	*positive*	X	X		X	X	
	2019	AO-Tek-2019_51	*A. uralensis*	*positive*	X	X		X	X	
West Kazakhstan region	2018	WKO-Bay-2018_20	*A. uralensis*	*positive*	X	X		*R. slovaca 99,44%*	*R. slovaca*	ON604649
		WKO-Bay-2018_23	*A. uralensis*	*positive*	X	X		X	X	
		WKO Bay-2018_26	*A. uralensis*	*positive*	*R. raoultii Isolate Tekeli 041 100%*	*R. raoultii*	ON604643	X	X	
		WKO-Bay-2018_39	*M. arvalis*	*positive*	*R. raoultii Isolate Tekeli 041 100%*	*R. raoultii*	ON604644	X	X	
	2019	WKO-Bay-2019_40	*A. uralensis*	*positive*	X	X		*R. conorrii strain 1450, 99,73%*	*R. conorii*	ON604650

X no sequence generated
*** Fragment to short for phylogenetic analyis
R. *Rickettsia*

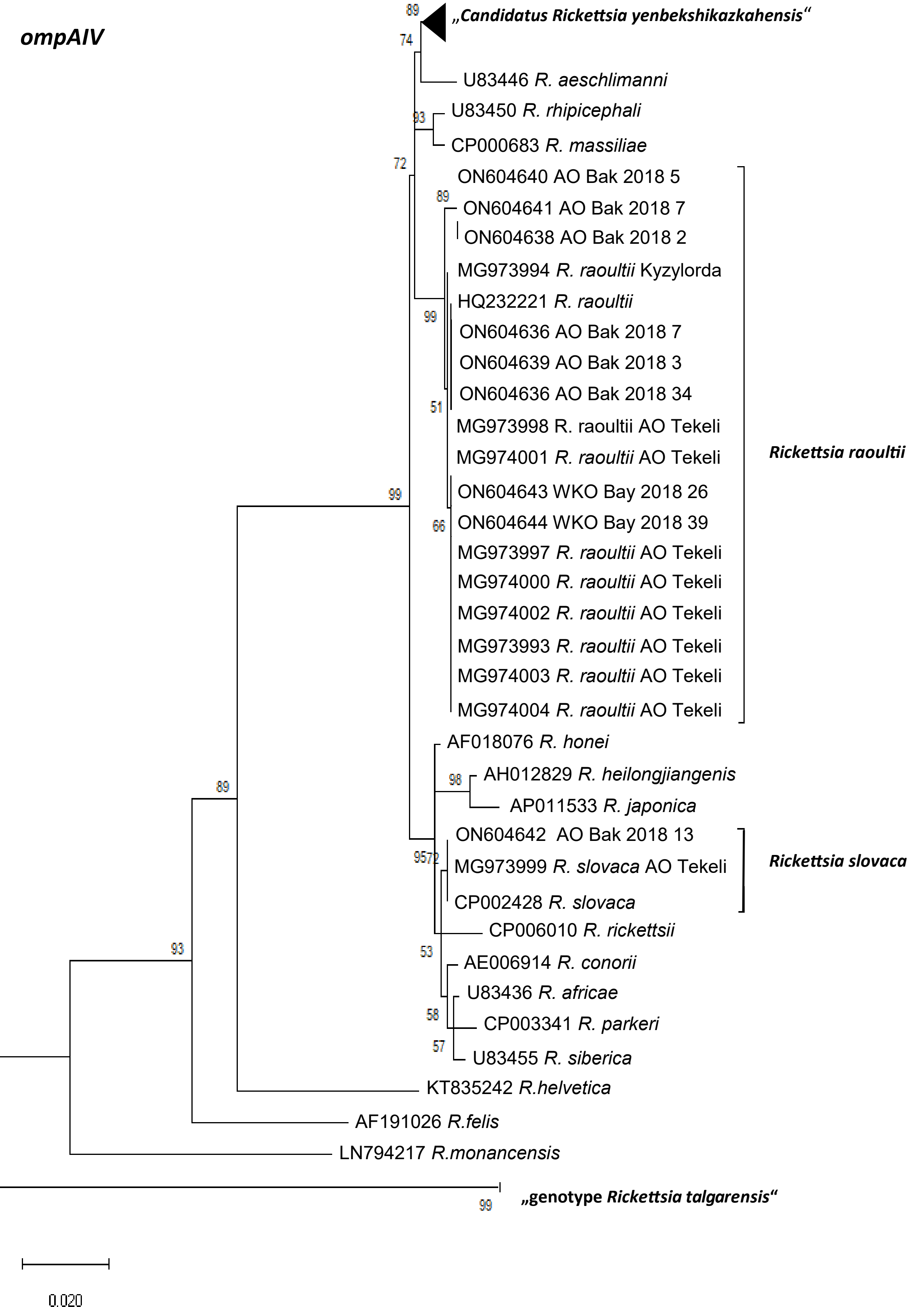

Figure 2: Maximum Likelihood phylogenetic tree based on 68 partial *ompAIV* DNA sequences. Nine sequences are originating from amplificates from small rodents from Kazakhstan and 59 from the GenBank database. Eight of the new generated sequenes from Kazakhstan were 99-100% identical to *R. raoulti* and one were 100% identical to *R. slovaca.* In addition, 30 sequences form the *Candidatus Rickettsia yenbekshikazkahensis* and three sequences form the „genotype *Rickettsia talgarensis*“ cluster. The tree with the highest log-likelihood (-2445.21) is shown. There are in total 720 positions in the final dataset.

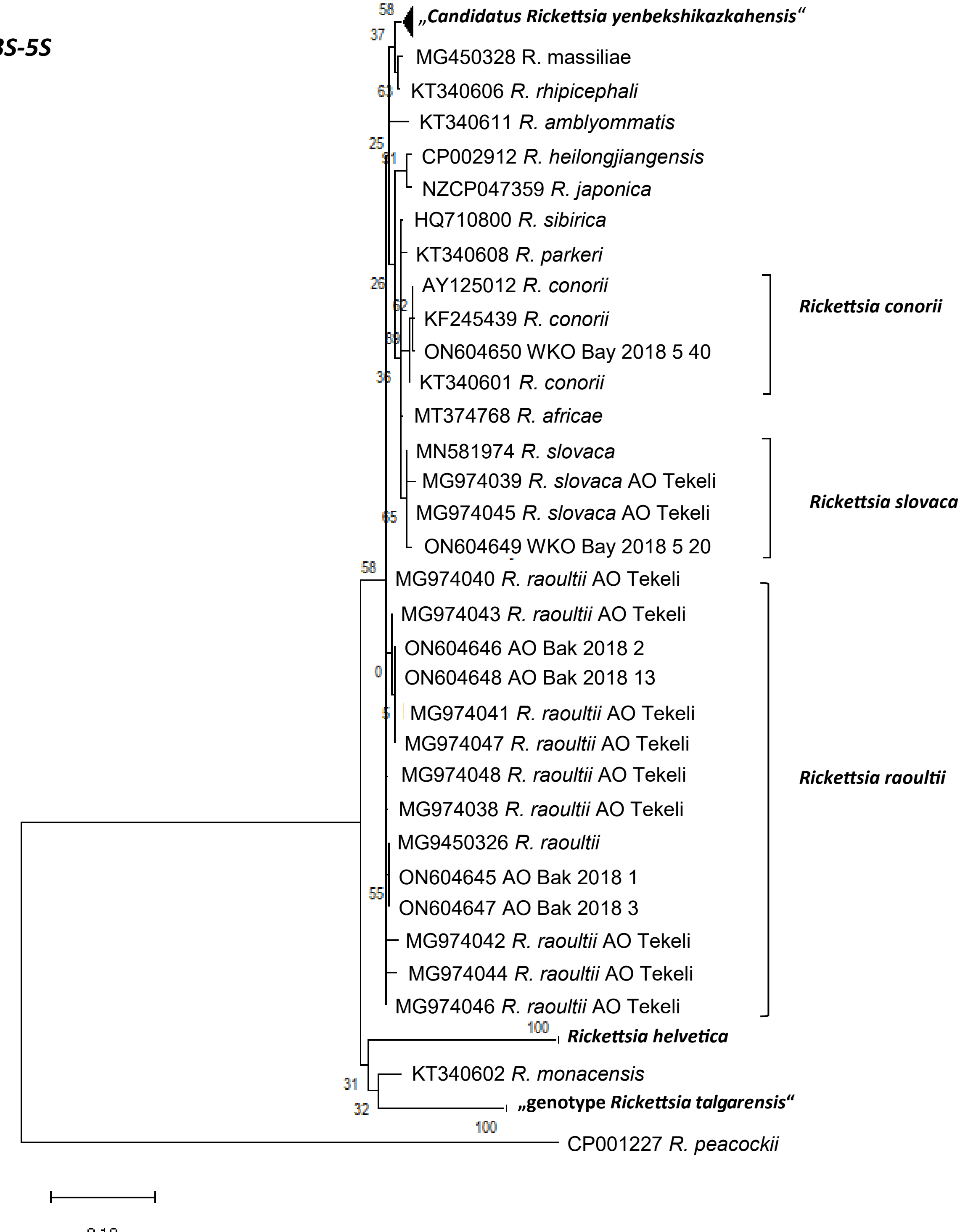

Figure 3: Maximum Likelihood phylogenetic tree based on 46 partial *23S-25S Interspacer* DNA sequences. Six sequences originating from amplificates from small rodents from Kazakhstan and 40 from the GenBank database. Four of the new generated sequenes from Kazakhstan were 99-100% identical to *R. raoulti,* one were 99% identical to *R. slovaca* and one were 99% identical to R. *conorrii.* In addition, nine sequences form the *Candidatus Rickettsia yenbekshikazkahensis* and two sequences form the „genotype *Rickettsia talgarensis“* cluster*.* Three sequences form the cluster of *Rickettsia helvetica. T*he tree with the highest log-likelihood (-1639.61) is shown. There are in total 411 positions in the final dataset.

Supplemental table 1: Overview of collected small mammals, sampling sites, captured numbers and numbers of positive specimen.

Region	Year	sampling region	Sampling site	Rodents total	Family of *Cricetidae*			Family of *Muridae*				Family of *Gliridae*	Insectivores		Rickettsia positive of sampling site n (%): Realtime RT-PCR	Rickettsia in species
					Mircrotus arvalis	*Clethrionomys glareolus*	*Microtus kirgisorum*	*Apodemus uralensis*	*Mus musculus*	*Rattus norvegicus*	*Meriones meridianus*	*Dryomys nitedula*	*Crocidura suaveolens*	*Sorex spp.*		
Almaty region	2018	Almaty region	Bakanas	15	0	0	0	0	13	0	2	0	0	0	**9 (60)**	*M. musculus* (8); *M. meridianes* (1)
			Tekeli	75	14	0	0	41	8	0	0	11	0	1	**2 (2.7)**	*M.arvalis* (2)
		Almaty City	Flooplain small Almatinka	20	0	0	1	5	8	1	0	0	5	0	0	
			Stroikombinat	12	1	0	3	0	3	1	0	0	4	0	0	
			Railway	9	0	0	1	5	0	0	0	0	3	0	0	
			Airport	27	0	0	0	5	2	12	0	0	8	0	0	
			Mercur	12	0	0	0	9	0	0	0	0	3	0	0	
			Autopark	7	0	0	0	6	0	0	0	0	1	0	0	
	2019	Almaty region	Tekeli	80	29	0	0	43	6	0	0	2	0	0	**1 (1.3)**	*A. uralensis* (1)
			Rudnichniy	30	30	0	0	0	0	0	0	0	0	0	0	
		Almaty City	Airport	35	0	0	2	5	6	19	0	0	3	0	0	
			Railway	39	0	0	22	6	9	0	0	0	1	1	0	
			Mercur	14	0	0	0	2	6	6	0	0	0	0	0	
			Tropinka	29	0	0	20	4	5	0	0	0	0	0	0	
West-Kazakhstan region	2018	WKO	Bayterek	58	7	3	0	45	0	0	0	2	0	1	**4 (10)**	*A. uralensis* (3); *M. arvalis* (1)
			Borili	10	0	0	0	0	10	0	0	0	0	0	0	
			Oral	27	1	0	0	26	0	0	0	0	0	0	0	
			Taskala District	16	0	0	0	0	16	0	0	0	0	0	0	
			Teretki	9	0	9	0	0	0	0	0	0	0	0	0	
	2019	WKO	Bayterek	48	0	1	0	36	10	0	0	0	0	1	**1 (2.1)**	*A. uralensis* (1)
			Borili district	15	0	0	0	5	10	0	0	0	0	0	0	
			Oral	10	0	0	0	10	0	0	0	0	0	0	0	
			Teretki	27	5	0	0	6	16	0	0	0	0	0	0	
			Total	**624**	**87**	**13**	**49**	**259**	**128**	**39**	**2**	**15**	**28**	**4**	**17 (2.72)**	

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Frau Prof. Dr. Ehrhardt, Frau PD Dr. Essbauer, Herr Dr. Peintner, Frau Nur Tukhanova und Frau Anna Shin,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum Unterschrift des Verfassers

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Appendix *Curriculum Vitae*

Publications

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Shin, A., Tukhanova, N., Ndenkeh, J., Shapiyeva, Z., Yegemberdiyeva, R., Yeraliyeva, L., Nurmakhanov, T., Froeschl, G., Hoelscher, M., Musralina, L., Toktasyn, Y., Gulnara, Z., Sansyzbayev, Y., Aigul, S., Abdiyeva, K., Turebekov, N., **Wagner**, **E**., Peintner, L., Essbauer, S.. Tick-borne encephalitis virus and West-Nile fever virus as causes of serous meningitis of unknown origin in Kazakhstan. *Zoonoses and Public Health* 2022, 00, 1– 12. https://doi.org/10.1111/zph.12941

Peintner L.*, **Wagner E.*,** Shin A., Tukhanova N., Turebekov N., Abdiyeva A., Spaiser O., Serebrennikova Y., Tintrup E., Frey S., Dmitrovskiy A., Zhalmagambetova A., Essbauer S.. Eight years of collaboration on biosafety and biosecurity issues between Kazakhstan and Germany under the German Biosecurity Programme and the G7 Global Partnership Against the spread of Weapons and Materials of Mass Destruction, Front Public Health. *Front. Public Health*, 2021. https://doi.org/10.3389/fpubh.2021.649393 (*contributed equally)

Abdiyeva K., Turebekov N., Dmitrowsky, A., Tukanova N., Shin A., Yeralieyeva L., Heinrich N., Hölscher M., Yegemberiyeva R., Shapieyeva Z., Kachiyeva S., Zhalmagambetova A., Montag J., Dobler G., Zinner J., **Wagner E.,** Frey S., Essbauer S.. Seroepidemiological and molecular investigations of infections with Crimean-Congo hemorrhagic fever virus in Kazakhstan. *International Journal of Infectious Diseases* 2019. https://doi.org/10.1111/zph.12683

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Tukhanova, N., Shin, A., Abdiyeva, K., Turebekov, N., Yeraliyeva, L., Yegemberdiyeva, R., Shapiyeva Z., Froeschl, G., Hoelscher, M., **Wagner, E.**, Rösel, K., Zhalmagembetova, A., Musralina, L., Frey, S., Essbauer, S.. Serological investigation of orthohantaviruses in patients with fever of unknown origin in Kazakhstan. *Zoonoses Public Health*. 2020; https://doi.org/10.1111/zph.1268.

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