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Characterization of tick-borne encephalitis virus in Kazakhstan by serological and molecular-biological techniques

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Key Words

Zoonosis, Tick-borne encephalitis virus, West Nile Fever virus, meningitis, serology, tick, Siberian subtype

Abstract

Tick-borne Encephalitis virus (TBEV), leading to one of the most dangerous neuroviral infection in humans named Tick-Borne Encephalitis (TBE) and West Nile fever virus that is a cause of neurotropic infection West Nile fever (WNF) are both belonging to the viral family of flaviviruses. In the Republic of Kazakhstan only limited data about TBEV, TBE and WNFV are available. In the last ten years 348 cases of TBE were registered but they are based on unreliable serological investigations. Furthermore, in 400 cases between 2017-2019 the aetiological agent of serous meningitis could not be determined and hence they were declared as cases of serous meningitis with unknown origin. TBEV is transmitted by ticks and up to date there is hardly any information about the rate of infected ticks in Kazakhstan.

The presence of WNFV in the Republic of Kazakhstan was confirmed in mosquitos and by the presence IgG specific antibodies in human sera in West Kazakhstan. However, there is no official registration of WNF cases or monitoring of mosquitos and/or birds as vectors.The similarity of the antigenic structure of the members of the flavivirus leads to the need for reliable diagnostic methods to discriminate between these pathogens. In our study we investigated three regions of Kazakhstan with TBE cases that are East Kazakhstan Oblast, Almaty and Akmola Oblast including 166 sera and 130 cerebrospinal fluid (CSF) samples from patients with meningitis symptoms. In addition, participants needed to answer a questionnaire with focus on socio-demographic factors, travel history, contact to animals and vectors, tick bites, vaccination status and clinical symptoms to understand more about the mode of infection.

All samples were tested for flaviviruses by screening for IgG and IgM antibodies in an ELISA, an immunofluorescence assay using a flavivirus biochip and a TBEV specific real-time RT-PCR. Our findings show TBEV and WNFV antibodies in 31 samples.

In Kazakhstan also only limited data on the phylogenesis of TBEV is available. Hence, TBEV in its tick vector was also investigated in our studies. In more than 10,000 screened ticks from the three regions 0.5% of pools were found to be positive for TBEV using a specific real-time RT-PCR. Phylogenetic analysis of gained sequences showed the Siberian subtype of TBEV to be predominant in Kazakhstan.

In this study, we present a broad investigation of the spread of TBEV in Kazakhstan in humans and also in ticks. Our results have an impact on diagnostic algorithms in Kazakhstan and medical doctors should be aware of TBEV and WNFV to play a role in meningeal diseases of unknown origin in the Republic of Kazakhstan.

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List of abbreviations

AkO	Akmola oblast
APS	Anti-plague station
AO	Almaty oblast
EKO	East Kazakhstan oblast
ICTV	International Committee for Taxonomy
IFA	Immunofluorescence assay
Ig	Immunoglobulin
KAZ	Republic of Kazakhstan
NCPHC	National Centre of Public Health Care of the Ministry of Health of the Republic of Kazakhstan
NKO	North Kazakhstan oblast
NT	Neutralization test
PCR	Polymerase chain reaction
RNA	Ribonuclease acid
RSSE	Russian Spring Summer Encephalitis
CNS	Central nervous system
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
WNFV	West Nile fever virus
WNF	West Nile fever

1. Introduction

The virus family of Flaviviridae includes about 50 serologically related viruses (Simmonds, 2017). This family is spread worldwide and is geographically dispersed by the migration of birds, transportation of livestock or human travelling. Human infections with different species of Flaviviridae are often challenging to differentially diagnose by doctors, since their symptomatology is frequently similar, and the antigenic vicinity between the species leads to cross reactions in commercially available assays with a resulting amplified likelihood of false-positive tests (Rathore and St John, 2020).

1.1 Tick-borne encephalitis virus

Tick-borne encephalitis virus (TBEV) is an affiliate of the genus flavivirus within the family of Flaviviridae. Most members of this family belong to the ecological group of arboviruses, *i.e.* they are transmitted by mosquitoes or ticks. In 1937 Russian virologists found out that Russian spring summer encephalitis (RSSE), is a virus infection, transmitted by the tick species *Ixodes persulcatus* (Zilber, 1962). During that time, they also developed the first vaccine against RSSE (Tsurupa, 1940). In 1939 another Soviet scientist Pavlovsky and others described zoonotic transmission between ticks and mammals (Karpova, 2012). For the first time, in Central Europe, tick-borne encephalitis virus was isolated by Gallia in Bohemia in 1948 and the infection was named back then as Central European encephalitis (Kunz, 1992). In 1989 Pletnev and others decoded the full genome of Russian TBEV strains (Pletnev et al., 1989). This work has greatly contributed to the study of the molecular biology of tick-borne encephalitis virus, made it possible to study the genome variability and virulence, to develop a tick-borne encephalitis virus specific polymerase chain reaction (PCR) analysis and to clarify the pathogenesis, the clinical and the epidemiological manifestations of TBE. Scientists such as G. Dobler, W. Mandl, F.X. Heinz, C. Kunz, N. Holzmann, E.K. Pressman, N.A. Tsekhanovskaya, L.E. Matveev, A.A. Kushch, and others worked on the study of monoclonal antibodies to the main antigens of the tick-borne encephalitis virus and the related family. These works made it possible to apply monoclonal antibodies to study the immunogenic, pathogenic and other properties of the structural and non-structural proteins of tick-borne encephalitis virus and to develop an efficient molecular diagnosis for tick-borne encephalitis (Ammosov, 2006).

1.1.1 Tick-borne encephalitis virus epidemiology

TBEV is endemic from central Europe through the Eurasian continent reaching till Far East Asia. TBE cases were registered so far in Albania, Austria, Belarus, Bosnia, Byelorussia, Bulgaria,

China, Croatia, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Japan, Kazakhstan, Latvia, Lithuania, Moldavia, Mongolia, Norway, Poland, Russia, Romania, Serbia, Slovakia, Slovenia, Sweden, Switzerland, Turkey, and Ukraine (Kaiser, 2008). The main hosts and reservoirs of the TBEV are small rodent species (voles, wild mice, and shrews). Ticks act as the vector, and humans are accidental hosts. The TBEV transmission within the main host population can occur by two ways: (i) vertical (*i.e.* from ova to larvae and from larvae to nymph and to imago) and (ii) horizontal (*i.e.* from tick to tick, from tick to vertebrate host, or vice versa) (Moshkin et al., 2009). Ticks can obtain TBEV while they suck blood from an infected host. Further, ticks infect their hosts through the saliva secreted during feeding (Filippova, 1985).

1.1.2 Tick-borne encephalitis vectors

More than 130 species of mammalian wild and domestic animals and birds are reservoirs and serve as sources of transmission of the TBEV. However, the main host species are *Ixod*es ticks, which sustain the pathogen in nature. Spontaneous infection with the TBEV has been established in 16 species of *Ixodes* ticks. There is evidence that tick species such as *Ichthyococcus ovatus*, *Ixodes nipponensis*, *Dermacentor marginatus, Haemaphysalis punctata*, *Haemaphysalis japonica*, *Haemaphysalis phasiana*, among others, are also vectors of TBEV (Cisak et al., 2010, Yu et al., 2015). These tick species occur primarily in China, Russia, Kazakhstan and Kyrgyzstan (Shapieva Zh. Zh. et al., 2008). Out of these, only two species are categorised as major vectors, namely the taiga tick (*Ixodes persulcatus*) and the forest tick (*Ixodes ricinus*), which are distributed in the forests and meadows of almost all European countries, in European Russia and Siberia, as well as in seven Asian countries (Jaenson et al., 2012, Popov and Harit, 2011). *Ixodes ricinus* is the main vector for European strain of TBEV strain also dubbed TBEV-Eu (Valarcher et al., 2015), while the other two TBEV subtypes, Siberian TBEV and Far East TBEV, are transmitted mainly by *Ixodes persulcatus* (Chicherina et al., 2015). TBEV can also be transmitted by tick bites from *Dermacentor spp*. (Süss, 2011). Further in Europe and in the Western part of Russia the main vector of TBE is *I. ricinus* while in Siberia and Central Asia the *I. persulcatus* prevails.

The infestation of the vectors by virus is varying. In Western Europe only 1-2% of ticks are infected with TBEV, while from Siberia tick infection rates up to 38% are reported (Jelinek, 2012). Ticks can be infected or infect the host while they are suckling blood from their hosts (Moshkin et al., 2009). The natural hosts of TBEV are mainly rodents (Weidmann et al., 2011). TBEV circulates between ticks and hosts in geographically strictly limited natural foci, which can range in size from large to very small (Dobler et al., 2011). Humans are only accidental hosts of TBEV because they are a dead end of virus circulation (Gritsun et al., 2003). To humans the virus can be either transmitted by tick bites or by consumption of unpasteurized milk or raw milk products (Nurmakhanov et al., 2013).

1.1.3 Tick-borne encephalitis virus taxonomy

According to the International Committee for Taxonomy (ICTV) of viruses, TBEV is divided into three subtypes (King et al., 2012): (i) The European subtype (TBEV-Eu), endemic in rural and forest areas of central, eastern and northern Europe; (ii) the Far Eastern subtype (TBEV-Fe) known as RSSE, virus is circulating in the Eastern part of Russia and Japan; (iii) the Siberian Subtype (TBEV-S) circulating in Siberia, Central Asia and Korea. In the last years two more subtypes have been detected – Baikalian (TBEV-Bkl) (Kovalev and Mukhacheva, 2017) that was found in East Siberia near Baikal Lake and Northern Mongolia and is remarkable by its in-between phylogenetic position among TBEV-Sib and TBEV-FE, and Himalayan subtype (TBEV-Him) (Dai et al., 2018) which was discovered in wild rodent *Marmota himalayana* in Qinghai-Tibet Plateau in China.

1.1.4 Tick-borne encephalitis virus microbiology

TBEV is belonging to the group of RNA arboviruses. The virus is characterized as a small, enveloped particle containing a positive sense single-stranded RNA containing roughly 11 kilobases (kb). The virus is generally sensitive to environmental factors and it dies rather rapidly at room temperature, when heated to 60°C for more than 10-20 minutes or after 2 minutes of boiling. It is rapidly destroyed by disinfectants or UV light. However, it is resistant in milk that is not high heated or pasteurised, it can retain there up to 2 months and in a dried state, it can even exist for years. The genomic RNA contains one open reading frame, which is flanked by 5′ and 3′ untranslated regions and encodes a polyprotein of about 3400 amino acids that is cleaved into three structural proteins (C protein (capsid), prM/M protein (membrane and its precursor) and E protein (envelope)), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by cellular and viral proteases. This E protein is responsible for essential functions during virus entry including receptor binding and membrane fusion and it induces neutralizing antibodies and protective immunity (Patrice et al., 2015).

1.1.5 Tick-borne encephalitis: clinical aspects

TBEV is the causative agent of tick-borne encephalitis (TBE), a potentially deadly central nervous system (CNS) infection in man (Monath, 1990). The first description of TBE was found in church registers in the 18th century in Finland (Gritsun et al., 2003). The scientific investigation of TBE has started in 1927 when 24 cases of aseptic meningitis were described by the Austrian physician Schneider (Lange et al., 2021). TBE is endemic in Central and Eastern Europe, in Russia and in Asian countries and up to 3,000 cases of TBE are registered in Europe each year and in Russia up to 10,000 cases (Süss, 2011). TBE is characterized by a polymorphism of clinical manifestations and severity of forms. The incubation period is 4 to 28 days, on average 7-14 days

(Ammosov, 2006). About a third of infected and symptomatic patients develop subsequently the a of tick-borne encephalitis (Kaiser, 2008). This encephalitis appears usually in a biphasic presentation. In the first viraemic phase the patient develops nonspecific flu-like symptoms like fever, headache, myalgia, some gastrointestinal symptoms, leuko- and thrombocytopenia in blood tests and increasing of levels of the liver related enzymes. The second phase is characterized by the involvement of the Central nervous system with a development of possible severe meningitis or meningoencephalitis (Lindquist and Vapalahti, 2008). The severity of TBE can range from mild to very severe courses with fatal outcomes. The severity of the course is depending on several factors such as age of the patient and the viral subtype (Kaiser, 2008).

In general, five TBE clinical forms can be distinguished (Kiffner et al., 2010), (Yegemberdiyeva et al., 2013):

1. febrile form
2. meningeal form
3. meningoencephalitis form
4. poliomyelitis form
5. polyradiculoneuritic form .

The **febrile** form is the mildest phenotype, with an incidence rate of 40-50% (sometimes up to 70%, depending on the region) (Yushchuk N. D. and Vengerova, 2009). It is is characterized by high fever. Additional general cerebral symptoms include headache, dizziness, nausea, and possibly even vomiting. Rarely, meningism may occur, the meningism is a syndrome with positive meningeal signs but without inflammation of CNS. The fever lasts on average five to seven days, dropping by day nine or ten. Rarely, the febrile period may be shortened to three to four days, indicating a mild course of the disease. The febrile form is the most favourable regarding the prognosis. It usually ends with fully recovery.

The **meningeal** form is the most common, occurring at an average of 50-60% (Yushchuk N. D. and Vengerova, 2009). The severity of meningeal symptoms depends on the severity of the disease. The clinical picture is characterized by headache of varying intensity and localization, pain in the eyeballs, nausea, and vomiting. The first days of the illness are determined by the stiffness of occipital muscles and Kernig's sign, it is a test used to look for evidence of irritation of the meninges. The test involves flexing the thighs at the hip, and the knees, at 90-degree angles, and assessing whether the subsequent extension of the knee is painful, in which case it is deemed positive. The meningeal symptoms may occur with drowsiness and psychomotoral agitation in the severe forms. Some patients present with diffuse neurological symptoms indicating the involvement of individual cranial nerves and brain parenchyma (*e.g.* facial asymmetry, mild deviation of facial asymmetry, mild deviation of the tongue, mild absence of outward movement of the

eyeball, animation or suppression of tendon reflexes, and anisoreflexia). The meningeal symptoms regress after 8-20 days but can last up to two months, leaving a lasting cerebral aggravation and intracranial hypertension. When performing spinal puncture, the cerebrospinal fluid shows inflammatory changes that are characteristic of serous meningitis. Pleocytosis is predominantly lymphocytic, ranging from a few to a few hundred cells, but sometimes reach 1,000 cells/ml. In the first days of the disease, the cytosis is of mixed or neutrophilic character, but by the end of the first week, it develops into a defined lymphocytic character. Beside the traces of lymphocytes in the CSF the protein content becomes moderately elevated up to 0.66 g/l. The content of glucose in CSF is normal. In rare cases, hyperglycoraxia and hypochloridoraxia are observed. Recovery of the CSF to normal values is observed within 3-5 weeks, sometimes pathological changes in the cerebrospinal fluid persist up to several months.

TBE in the more severe **meningoencephalitis** form is characterized by a severe course and high mortality. It can occur with both diffuse and focal cerebellar damage. This form of TBE occurs in 5-15% (Yushchuk N. D. and Vengerova, 2009) and is also characterized by high fever that is resistant to antipyretics. General cerebral and meningeal symptoms are followed by lethargy or syncope, and focal symptoms may include a spastic limb paresis and a paresis of the facial nerve, hyoid nerve and/or other cranial nerves. In rapidly increasing coma and seizure status, death may occur after two to four hours. The focal meningoencephalitis is characterized by the development of several movement disorders such as spastic hemiparesis, hyperkinesis, ataxia, akinesia, and the rigid syndrome. In contrast to peripheral movement disorders, patients developing a central paresis recover completely or only remain with small defects at the end of the acute period. The most severe cases are the Kozhevnikov's epilepsy syndrome, when permanent local myoclonias occur joins by hemiparesis and persistent localized myoclonias gradually spread from the distal extremities (more often the hands) to the proximal extremities, then to the face and the entire paretic half of the body. Further, myoclonias periodically intensifiy sharply and develop into a local or general epileptic seizure. Rarely, an amiostatic syndrome occurs as movement disorders, manifested by poverty and slowing down of active movements and a peculiar increase in muscle tone, with further regression within four to six weeks. In some cases, the only manifestation of the meningoencephalic form may be some cerebellar disorders, clinically manifested by dizziness, vomiting, nystagmus, ataxia, muscle weakness and intentional tremor. The outcome of the meningoencephalitis form contains residual effects in the form of mental disorders, long-term cerebral asthenia, decreased memory and intellect. Formation of a tetanic epilepsy is possible.

Polyomyelitis is one of the most severe of the TBE clinical forms. It has been reported in 1-2% of the patients with TBE (Yushchuk N. D. and Vengerova, 2009). Fever may persist in the patient for up to one month. This is accompanied by flaccid paralysis of the muscles located to the neck and upper extremities. Secondary bulbar disorders and cerebral edema are possible. Further

there are symptoms like the inability to hold the head, paresis in the upper limbs with subsequent development of atrophy of certain muscle groups of the shoulder girdle, chest and upper limbs. Death may occur within five to seven days of the onset of neurological symptoms (Yegemberdiyeva et al., 2013).

The **polyradiculoneurotic** form is also a focal form of TBE and occurs in only 1-3% of patients. In addition to the meningeal symptoms, an involvement of spinal and peripheral nerves occurs in the form of paresthesia, sensory disturbances in the distal limbs, and straight leg raise.

Due to the non-specific febrile form of the TBE, in which there are no signs of CNS damage, tick-borne encephalitis may not be verified in some individual cases. As a consequence, statistically recorded cases do not reflect the true incidence rate of the disease. An asymptomatic form of tick-borne encephalitis with a short febrile period is also known. Also, chronic forms of tick-borne encephalitis occur in 1-3% of patients. The formation of a chronic state can be explained by the long-term persistence of TBEV and the presence of virus-specific antigens in the organs and specific antibodies in the blood serum. Persistance of TBEV was found to be predominantly localized in the central nervous system and the spleen. Such latent infections may are caused by any external or internal (intercurrent diseases) exposure. Further, viral particles induce the process of demyelination in the CNS due to the activation of the T-helper system. (Khafizova I. et al., 2013). The chronic form of the disease develops in patients after acute tick-borne encephalitis, against a background of well-being after an indefinite period of time (from several months to several years), when clinical symptoms of the nervous system damage appear or develop with a steadily progressive or remitting course, which can lead to various degrees of disability or even death (Ammosov, 2006).

In general, in different regions the mortality rate ranges from 1% to 20% mostly depending on the subtype of TBEV circulating in the respective regions (Barrett et al., 2003). About 25% of the patients with neurological symptoms completely recover within two months but almost half of cases show permanent neurological or neuropsychological sequelae. TBE can also develop into long-term sequelae (Veje et al., 2016) that includes enduring neuropsychological symptoms, headache, ataxia, paresis and muscle atrophy (Karelis et al., 2012).

1.1.6 Tick-borne encephalitis diagnosis, treatment, and prophylaxis

Over the years several diagnostic methods were developed to identify an TBEV infection in patients. There are enzyme-linked immunosorbent assays (ELISA) that identify the circulation of IgM or IgG type antibodies reactive for TBEV (Girl et al., 2020). Furthermore, there is a TBEV-SNT as a confirmation test. Additionally, there is the method of comparative immunofluorescence assays, so called IFA Biochips (Mosaic 3, Euroimmun®, Germany). In recent years, real

time reverse transcription (rtRT) PCR was developed and is now one of the gold standards to identify an acute infection by TBEV.

The standard laboratory diagnosis of TBEV to screen human patients is carried out by ELISA with the determination of IgM and IgG antibodies in serum and CSF (Jelinek, 2012). More informative results are derived from the use of convalescent (paired) samples that can present a four-fold increase in IgG ELISA titres after *e.g.* fourteen days of immune reaction (Roelandt et al., 2017). However, an ELISA needs the confirmation by other means, since flaviviruses are highly cross-reactive and a previous vaccination against TBEV and previous infections with other flaviviruses may perturb the readout. Additionally, the IgM-seroconversion (the transition of IgM antibodies early after infection to IgG after about two weeks of infection) may not even develop in some patients (Kollaritsch et al., 2012). For the confirmation tests diagnostic laboratories either use an immunofluorescence assay (IFA) or a neutralization test (NT) (Venturi et al., 2006). Moreover, in the first five days of onset of the TBE related symptoms the antibodies do not even appear in the in serum and so the only way to identify the virus as causative agent of disease is the execution of a RT-PCR.
In European countries there is no specific treatment for TBE, therefore only symptomatic treatment is applied. The volume of therapy measures is determined by the complex of symptoms that the patient present (Ruzek et al., 2019).

In the Republic of Kazakhstan, as in some other former Soviet states, there is an application of a TBEV specific immunoglobulin standard therapy upon the suspicion of TBE. This specific anti-TBEV immunoglobulin is produced from the plasma of donors and is used for post-exposure prophylaxis and treatment in Russia and Kazakhstan (Pen'evskaia and Rudakov, 2010). This human derived medication is used for emerging prophylaxis after a tick bite and for treatment. However, in Europe outside Russia, this specific anti-TBEV immunoglobulin is not used.

The best form to protect individuals from infections with TBEV is a vaccination. Currently, there are six TBEV vaccines available for the prevention of TBE. Two vaccines are licensed in Europe, FSME Immun®, by Pfizer and Encepur® distributed by GlaxoSmithKline. The first European vaccine was prepared from seed virus, the so-called Neudoerfl (a small village located near Vienna, Austria) strain of the European subtype, isolated from ticks (Barrett et al., 2003). The second vaccine, Encepur®, is based on the Karlsruhe (a city in south western Germany) (K23) strain (Harabacz et al., 1992). Both vaccines also have paediatric formulations. The standard schedule of European vaccines is a shot of the first two doses 1–3 months apart followed by a third dose 5–12 months (FSME-IMMUN) or 9–12 (Encepur) later.

Three further vaccines are produced in the Russian Federation. They are named “TBE vaccine Moscow” and “Tick-E-Vac” and are both produced in Moscow. The “EnceVir®” vaccine is produced by the company Microgen in Tomsk. The vaccines produced in Moscow are based on the Sofjin strain (Vorovitch et al., 2020) and EnceVir® on the Far Eastern subtype, strain 205 (Safronov et al., 1991). All vaccines have special paediatric formulations. The vaccination schemes of the Russian vaccines TBE-Moscow and Tick-E-Vac are two shots one to seven months apart, and a first booster after 12 months with following boosters every three years. The EnceVir scheme consists of second dose administered five to seven months after the first dose, a first booster after 12 months and the following boosters every three years (Ruzek et al., 2019).

One vaccine against TBEV is produced in China. It is based on TBEV-FE strain Sen-Zhang, and is named SenTaiBao vaccine. The immunogenicity of this vaccine is low (Suleman et al., 2021).

1.2 West Nile Fever Virus (WNFV)

West Nile Fever virus (WNFV) is an additional member of the family of Flaviviridae, belonging to the genus *flavivirus*. Detected for the first time in Uganda in 1937, during the last 20 years its presence has been confirmed in several European and Mediterranean countries (Calistri et al., 2010).

1.2.1 West Nile Fever Virus epidemiology

WNFV was registered in Africa, Europe, the Middle East, North America and West Asia (Petersen et al., 2013). WNFV is circulating in a cycle between birds, mosquitoes and again birds. Humans and horses are incidental and so called “dead-end” hosts (Petersen et al., 2013). The endemic season is linked to the activities of the mosquitoes and usually lasts from July to September.

1.2.2 West Nile Fever Virus vectors

The main vectors of WNFV are mosquitos, where the *Culex* species is playing the predominant role (Campbell et al., 2002). Moreover, the presence of the WNFV in ticks has also been determined. The transmission to birds but also to dead end hosts happens during the blood feast of the parasite on their prey. However, there also reports that infections happened after a blood transfusions, organ transplantations or laboratory accidents that are also possible (Malkinson and Banet, 2002). Additionally, WNFV transmission can occur between an infected mother and its new-born through the intrauterine route (CDC, 2002) or by breast-feeding (Hinckley et al., 2007).

1.2.3 West Nile Fever Virus taxonomy

The WNFV species is grouped into two major genetic lineages. Samples from North, West and Central Africa, Southern and Eastern Europe, India and the Middle East have been combined in

lineage 1, and samples from West, Central and East Africa and Madagascar are grouped into lineage 2. Lineage 1 is further subdivided into three clades. Clade 1a consists of strains from Europe, Africa, the USA, and Israel. The Australian Kunjin virus belongs to clade 1b, whereas isolates from India form clade 1c (Lanciotti et al., 2002). However, the so called Rabensburg isolate 97-103 from *Culex pipiens* mosquitoes and LEIV-Krnd88-190 isolated from *D. marginatus* ticks are genetically different from lineage 1 and 2 viruses and have been proposed as members of lineages 3 (Rabensburg 97-103) and 4 (LEIV-Krnd88-190), respectively (Bondre et al., 2007, Lvov et al., 2004, Bakonyi et al., 2013).

1.2.4 West Nile Fever Virus microbiology

West Nile fever virus is spherical, 20-30 nm in size, contains a positive single-stranded unsegmented RNA and replicates in the cytoplasm of affected cells. The virion consists of an envelope surrounding an icosahedral capsid. The approximate 11 kb genome encodes a single open reading frame, which is flanked by 5′ and 3′ untranslated regions. The genome is first translated as a single polyprotein, and subsequent cleavage of this polyprotein by both, viral and host proteases, that generate three structural proteins required for the virion formation (capsid protein, C) and the assembly into a viral particle. This is supported by the premembrane protein (prM) and the envelope proteins (E). Furthermore, seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins are produced. The structural proteins form the virion that encapsulate the viral RNA and the non-structural proteins form the replication complex that is required for synthesis of negative- and then positive-sense viral RNA. Following the virion assembly and the subsequent transport through the host secretory pathway, the mature viral particle is released from an infected host cell by classical exocytosis (Rossi et al., 2010).

West Nile virus belongs to the antigenic complex of Japanese encephalitis, which also includes the pathogens of St. Louis encephalitis virus, Yellow fever virus, Dengue fever virus and others. The virus is resistant in the external environment; however, it also has its limits. It becomes ineffective if temperatures are above 55 ºC for at least half an hour. However, it remains functional when it is dried or frozen.

1.2.5 West Nile Fever Virus clinical aspects

The WNFV is the causative agent of West Nile fever that can develop as a neuroinvasive disease. The clinical presentation of WNF starts with the first phase of unspecific symptoms such as fever and myalgia. The second phase is characterised by the development of encephalitis with a potentially fatal outcome (Colpitts et al., 2012). However, WNF is asymptomatic in about 80% of infected people and, when symptomatic, most patients present a mild febrile disease (CDC, 2022). Separately from fever, the mild form of disease is manifested by several non-specific flu-

like symptoms (headache, fatigue, myalgia, arthralgia, weakness, rash, and gastrointestinal problems, including nausea and vomiting). Less than 1% of infected people develop severe WNF neuroinvasive disease. Those severe symptoms usually appear between two to 15 days after infection and last for up to five days. The above-mentioned symptoms of the second phase include meningitis, encephalitis, and poliomyelitis. Patients with encephalitis usually present movement disorders, including severe tremors and parkinsonism. The poliomyelitis can manifest as a poliomyelitis-like acute flaccid paralysis, which at its most severe presentation can cause quadriplegia, *i.e.* a paralysis of all limbs of the body, and respiratory failure with an estimated 10% of the neuroinvasive cases resulting fatal. Recovery from severe illness might take several weeks or months, and more than 50% of survivors that presented severe symptoms reported physical and cognitive sequelae up to 2 years later. Non-neurological clinical manifestations are less frequent. Secondary symptoms have been described and include ocular pathologies upon WNF, mainly bilateral multifocal chorioretinitis, but also vitritis, optic neuritis, retinal haemorrhage, and iridocyclitis. Likewise, hepatitis, pancreatitis, orchitis, myositis, or myocarditis are considered infrequent manifestations of WNF and are correlates of viraemia (Saiz et al., 2021).

1.2.6 West Nile fever virus diagnostics, treatment, prophylaxis

A diagnosis for WNF is mostly conducted by the detection of the specific IgM antibody in serum and cerebrospinal fluid (CSF). However, unfortunately specific IgM antibodies may not be detected in the serum at the time of hospitalization when the patient either gets admitted to the hospital too early before IgM antibodies are forming, but sometimes also too late when the class switch from IgM to IgG already occurred. Real time RT PCR for the detection of WNFV RNA is also available, however also here the exact timing of the sampling from the infected patient is important, since the virus resides in the patient for only eight to ten days (Petersen et al., 2013). WBFV treatment is symptomatic and is basically identical to the treatment of TBE, which can be explained by the high similarity of the biology of this two virus species.

Injections with drugs like interferon, ribavirin, and steroids are used as a standard treatment of viral meningitis. However, there is no systematic evidence that these schemes are effective (Rossi et al., 2010). Officially, there is no available vaccine for humans yet available on the market. A vaccine for horses is available worldwide but not applied in KAZ since the area of KAZ is not yet officially endemic for WNFV.

1.3 Introduction into the Republic of Kazakhstan

The Republic of Kazakhstan (KAZ) is a landlocked huge country with a continental climate located in Central Asia and ranks ninth in terms of its territory size in the world, behind Russia, China, USA, Argentina, India, and Australia. The total area of the country is 2.7 million km^2 inhabited by a relatively small population of 18.6 million people (see figure 1). The territory of Kazakhstan is divided into 17 administrative units - 14 oblasts (= regions) and three cities of national importance (Almaty, Nur-Sultan and Shymkent). Nur-Sultan is the capital of the Republic of Kazakhstan since 1997, hosting a population of two million, located in Akmola oblast. Almaty is the former capital of KAZ, now it is the biggest city of the Republic with a population about three million people and located in the South-East, in Almaty oblast.

The ethnic composition of Kazakhstan is multiple and consists of more than 18 ethnicities. According to the 2021 population census, Kazakhs comprise 69.01% of the population, Russians 18.42%, and others 12.57%, including Uzbek, Korean, German and others.

Figure 1: Location of the Republic of the Kazakhstan on the world map.

Historically, Kazakhstan has been the cradle of many pathogens. Its vast steppes and forests, and the climate from hot and dry in the South to bitter cold on the North have favoured the development of infamous diseases such as the Plague, Anthrax, Brucellosis, and Tularaemia. There exists a vast amount of potential natural foci for the vectors or reservoirs of some of these diseases. For instance, ancient animal burial sites may cause spontaneous outbreaks of anthrax after heavy rains or landslides or can be unearthed by digging activities. To observe and fight potential outbreaks, local authorities in different oblasts of Kazakhstan started to establish anti-plague stations (APS) from 1914 to 1949. Soon APS became regional centres for epidemiological bio surveillance of all potential highly dangerous pathogens. These include tick-borne encephalitis

virus (TBEV), Crimean-Congo haemorrhagic fever virus (CCHFV), plague, orthohantavirus and others (Peintner et al., 2021).

Still, due to the massive size of the country and outdated diagnostic tools there are still blank spots regarding the spread and epidemiology of especially dangerous pathogens in some areas of Kazakhstan. For many pathogens already known to circulate in neighbouring countries no or only limited data is available (Wagner et al., 2022). Many reports of infections in Kazakhstan are based on incomplete clinical manifestation reports of infected patients rather than on molecular biological and serological diagnostics. Since many viral or bacterial infections only display relatively unspecific clinical symptoms it often leads to the problem of misclassification of diseases. It is suspected that many cases of infectious diseases caused by especially dangerous pathogens, such as Orthohantaviruses, TBEV, and CCHFV, go unnoticed in endemic and so-far non-endemic areas in Kazakhstan (Peintner et al., 2021).

1.3.1 Tick-borne encephalitis in the Republic of Kazakhstan

In the Republic of Kazakhstan (KAZ) the first description of a TBE case was made in 1935 and the first virus isolation from a human was in 1941 (Jumatov and Dmitrienko, 1961). Later in 1947, TBEV was also isolated from the tick *I. persulcatus* (Linetskaya, 1949). Today in KAZ 24 to 48 cases of TBE are registered annually.

Table 1: TBE cases in Kazakhstan 2012-2021 (NCPHC, 2011-2020).

Administrative territories	years										Total
	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	
Akmola oblast	3	0	4	6	2	8	3	3	6	2	37
Almaty oblast	5	6	8	10	12	4	11	7	1	2	66
East-Kazakhstan oblast	13	13	5	15	21	17	22	15	21	10	152
South-Kazakhstan oblast	0	0	0	0	0	0	0	0	0	0	1
Almaty city	12	8	11	6	12	6	5	5	0	0	65
Nur-Sultan city	0	0	0	0	0	0	0	1	0	0	1
Zhambyl oblast	0	0	0	1*	0	0	0	0	0	1*	2
Kostanay oblast	0	0	0	2*	0	0	1*	0	0	0	3
Pavlodar oblast	0	0	0	0	1*	0	0	0	0	0	1
North Kazakhstan oblast	0	0	0	0	0	0	3	4	4	9	20

Total	**33**	**27**	**28**	**40**	**48**	**35**	**45**	**35**	**32**	**24**	**348**

*- imported cases of TBE from other oblasts\countries

Officially, TBE is endemic in Almaty Oblast (AO) and East Kazakhstan Oblast (EKO). Since 2010 TBE cases also have been registered in a third oblast of KAZ, Akmola oblast (AkO), now reporting two to eight cases annually. From 2018 in the neighbouring region North Kazakhstan Oblast (NKO) also started an annual registration of TBE cases.

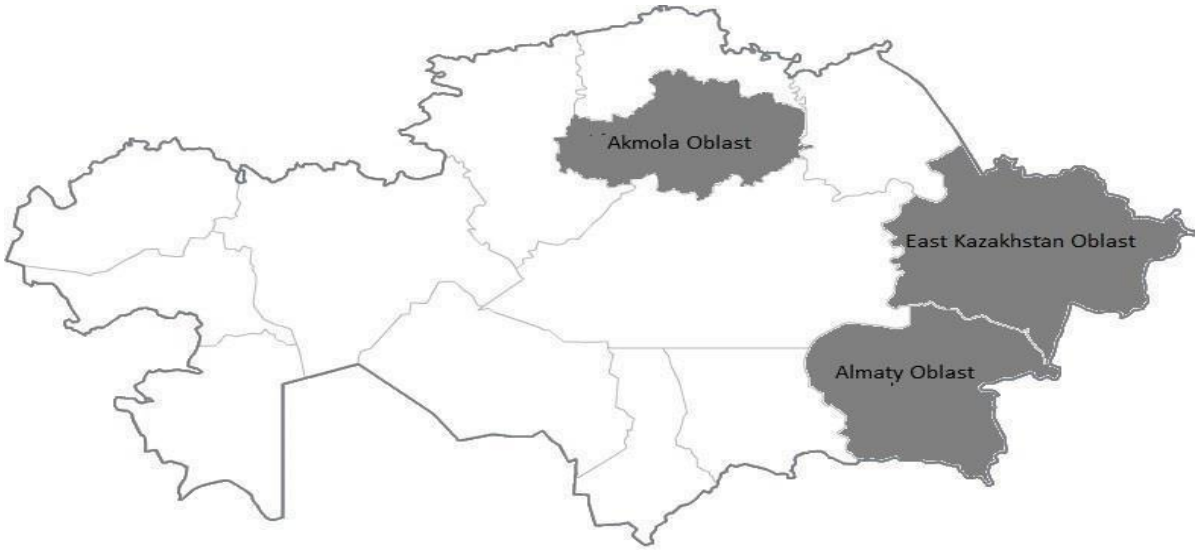

Figure 2: TBE endemic regions in the Republic of Kazakhstan.

Currently, the natural foci of tick-borne encephalitis in the Republic of Kazakhstan belong to the mountain-forest belt of the Tien Shan mountain range, the Dzhungarian and Zailian Alatau, Tarbagatai area, the valleys of Aksu, Tentek, Irtysh, and Ulba and the Bukhtarma rivers. Those regions are characterized by lush vegetation and high numbers of *Ixodes* ticks. More specifically, in these territories there are several independent endemic foci of tick-borne encephalitis, namely the villages Zyryanovsk, Katon-Karagay, Leninogorsk, Sarkand, Talgar, Enbekshikazakh and others, separated by often vast distances from each other. Due to the spread and different characteristics of the different hotspots, it is necessary to zonation the focal areas according to the degree of risk of infecting the population. In KAZ three types of foci are distinguished (Maykanov N.S. et al., 2012):

1. Type I – characterised by natural foci in wildlife
2. Type II - transient foci with changed composition of biocenosis components as a result of human activity;
3. Type III - anthropogenic (secondary) outbreaks in the vicinity of human settlements

The epidemiological surveillance of tick-borne encephalitis in the Republic of Kazakhstan is carried out on the basis of the Order of the Minister of National Economy of the Republic of Kazakhstan dated from 31 March 2015 (№ 283). This bill contains the approval of Sanitary Rules termed "Sanitary and Epidemiological Requirements to the Organization and Implementation of Sanitary and Anti-Epidemic (Preventive) Measures to Prevent Parasitic Diseases". Further it is backed by the Sanitary Rules named “On approval of epizootic surveillance monitoring for ixodid ticks order no. 56” dated a year later on 31 March 2016. According to these Sanitary Rules, the prevention of tick-borne encephalitis is carried out in the form of preventive and anti-epidemiological measures. Hence, the distribution, species composition, phenology, and number of ticks are annually studied by local sanitary epidemiological stations. However, the Sanitary Regulations do not assume the investigation of TBEV prevalence in ticks in endemic regions, nor do they stipulate the use of the molecular method (PCR) to determine TBEV contamination of ticks taken from affected humans and to diagnose TBE disease. Only the actual numbers of each tick species residing in defined areas are assessed.

Beside the surveillance of tick numbers, the annual human TBE cases are registered by the National Centre of Public Health Care belonging to the Ministry of Health of the Republic of Kazakhstan. Unfortunately, however, for the TBE diagnostical criteria in the Republic of Kazakhstan only ELISA screens for detection IgM against TBEV in acute patients is used and hence numbers are maybe not complete.

There is only limited data available about the characteristics of TBEV in KAZ, most data are only, as mentioned, based on sera-surveillance. Information about individual gene sequences as for instance from the E-gene or complete genomes for the territory of KAZ is completely absent. Furthermore, there is currently a lack of knowledge about the pathogenicity of the strains of TBEV circulating in KAZ. Only few efforts were published in a pilot study from Almaty Oblast (Abdiyeva et al., 2019). The only existent TBEV full genome registered in GenBank was made by a Russian scientist (L'Vov D et al., 2014). A more expanded knowledge of the nucleotide sequence of the TBEV genome and the resulting analysis of sequence identities and evolution distances would be a basis to learn more about the circulating subtypes and help to understand more about the virus virulence, the epidemiological situation and the correlation with the clinical manifestation of TBE. The necessity for this knowledge is especially important given the regular registration of TBE fatal cases.

The explanations for the scarcity in epidemiological data on TBE in KAZ are diverse, and include undefined symptomatology in the individual hospitals, lacking awareness in doctors and a very rudimentary testing capacity. Conferring to the National Centre of Public Health Care of the Ministry of Health of the Republic of Kazakhstan annual reports (NCPHC, 2011-2020) up to 400 cases of serous meningitis with unknown origin were registered each year in the three years of 2017-

2019. A part of those serous meningitis cases with unknown aetiology may be assumed to be unrecognized TBE cases (NCPHC, 2011-2020).

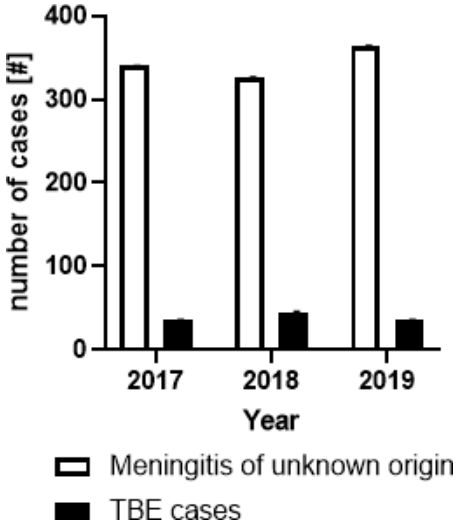

Figure 3: Serous meningitis of unknown origin and recorded TBE cases 2017-2019 in Kazakhstan. (Many cases of serous meningitis have an unknown aetiology (white bar). A tick-borne encephalitis virus infection induced meningitis is also frequently diagnosed (black bar). Image modified from (Shin et al., 2022).

1.3.2 West Nile Fever in Kazakhstan

In the Republic of Kazakhstan there is no official registration of WNF yet. However, it is known that WNFV is residing in the West Kazakhstan oblast (Maikanov, 2016). In this study it was found in migrating birds and mosquitos. Furthermore, the authors also identified traces of past WNFV infections in the sera of the local human population of the West Kazakhstan oblast. Some of the sampled humans developed specific IgG antibodies against WNFV. In order to declare a disease officially endemic in an oblast the KAZ regulations require actual observed infections in humans, being treated for symptoms in hospitals. Once such human infections are observed for several years, an emerging pathogen will be declared as officially endemic.

Figure 4. West Kazakhstan oblast is the region where the presence of WNFV was confirmed.

1.4 Reaction of the human immune system on a flavivirus infection

Due to the omnipresent nature of viruses, multicellular organisms developed a multitude of strategies to fight off potential harmful infections. In higher animals and vertebrates such as humans the immune system is after the tightness of the skin barrier the most important and versatile weapon against viral infections. An entering virus faces two antiviral strategies of the immune system, that is the innate immune system and the adaptive immune system.

1.4.1 Innate immunity

The first barrier that will meet viral pathogens is the so called innate immune system that is represented by several anatomical and chemical barriers, consisting of natural killer cells, macrophages, neutrophils, and dendritic cells that are constantly patrolling epithelial tissues. When an infection enters the body the specific pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) of these nucleated cells. That binding leads to the activation of a signal cascade leading to the production of interferon (IFN) (Kawasaki and Kawai, 2014), which plays a protective role in viral infections. Further, an infection modulates the patterns such as PRRs, cytokines, or chemokines that are expressed in the blood and solid tissues (Fares et al., 2020).

1.4.2 Adaptive immunity

The in the previous chapter addressed innate immune system works as a first barrier against a viral infection to give some time for the activation of the adaptive immune system. This adaptive immune system divided to two branches. The B- and T-cell mediated immune response. The B-cell or also humoral immune system is important for infection diagnosis. B cells can produce

specialised antibodies, that are able to neutralize virus infection and support the pathogen elimination in cooperation with the complement system (Reed and Muench, 1938). After the infection with a flavivirus, the amount of virus-specific antibodies in human serum and cerebrospinal fluid increases constantly and first antibodies are usually detectable above the sensitivity threshold with the onset of neurological symptoms.

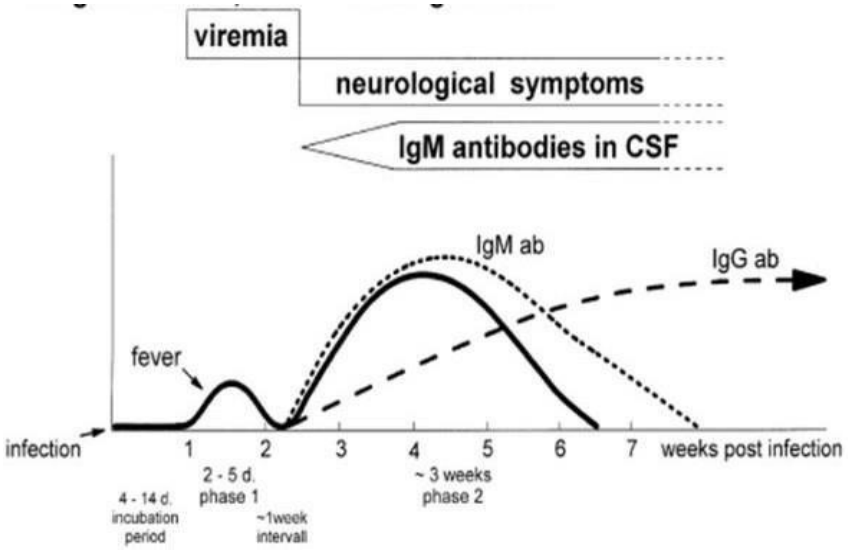

Figure 4: The antibody development during the flavivirus infection in serum and CSF. IgM ab: IgM specific antibody, IgG ab: Immunoglobulins of class G. Adapted from (Roelandt et al., 2017)

In the first days of the onset of the disease there are no reactive antibodies in the serum and the tissue of the infected patient, and the viral RNA can be detected in serum and CSF. Then, starting after four to five days the immunoglobulins of class M appear and can persist for around six weeks. The immunoglobulins of class G also start developing after fifth day of the onset of the disease but slowly increase with the IgG levels peak in the late convalescence period (Günther et al., 1997). This IgG antibodies can persist in serum from a couple weeks to even years (Jongejan F, 2013). For example, some studies on vaccine-induced antibodies showed a durability of virus neutralizing antibodies from five years to even ten years and more (Wittermann et al., 2009).

Interestingly, antibodies induced by a past flavivirus infection or vaccination may cross-react within the full antigen complex (McAuley et al., 2017) and with other flaviviruses (Stiasny et al., 2006). For example the vaccination with TBEV vaccine protected mice against Omsk haemorrhagic fever virus (OHFV) (Chernokhaeva et al., 2016). Moreover, sera from persons vaccinated against TBEV or a previous infection from TBEV could partially protect against Louping ill virus (LIV) and WNFV (Mansfield et al., 2011). So, these results indicate the presence of cross-reactive epitopes among flaviviruses and should be taken into account during the diagnostical tests.

1.5 Open questions on Flaviviruses in Kazakhstan

Tick-borne encephalitis virus is reported as a causative agent for one of the most important arbovirus infections that can lead neurological disorders for many years. However, there is only limited information about the spread of TBEV in the various regions (= oblasts) of Kazakhstan in its natural hosts and about strains existing on this territory. Furthermore, there are still many human cases of serous meningitis of unknown origin (MUO) that might be caused by an infection with a virus of the flavivirus family. Hence, we aim to investigate on the spread of TBEV in ticks and MUO patients to answer the following questions:

- What is the rate of TBEV infected ticks in selected regions of Kazakhstan?
- What is the genetic heritage of the viruses isolated? Is it possible to identify any migration patterns?
- What is the rate of flavivirus infection in patients with MUO?

This study was started with the aim to learn more about the role played by TBEV and other flaviviruses in human cases of serous meningitis of unknown origin in Kazakhstan in the regions of East Kazakhstan region, Akmola region and Almaty city. Additionally, we wanted to understand the sociodemographic and medical characteristics of infected patients to draw conclusions of potential risk factors typical for the area.

2. Material and Methods

This study was conducted as a cross-sectional study and divided into two parts, a serological investigation in human patients and a tick investigation. The serological part included sera and CSF investigation in three regions of KAZ for TBEV-specific antibodies in patients with suspected cases of serous meningitis. The tick investigation consisted of determination of a TBEV presence in tick population in endemic regions of KAZ with a following phylogenetic analysis.

According to the Risk Group Database of American Biological Safety Associations (ABSA) tick-borne encephalitis virus belongs to the 3-4 risk group, depending if collected in an endemic or in a non-endemic region. Thereby, work with material that could potentially contain tick-borne encephalitis virus should be carried out in a BSL3 laboratory.

2.1 Ethical approval

The study was reviewed and approved by the Kazakh National Medical University (opinion number #565) and by the Ludwig-Maximilians-Universität (opinion number #19-373) ethics committee. All participants were required to sign an informed consent. The text of the informed consent was translated into Russian and Kazakh languages since both are officially accepted in KAZ. The informed consent included the patient's permission for blood and CSF sampling, collection and processing of personal data and privacy guarantee.

2.2 Human seroprevalence study

2.2.1 Sample collection

The study was performed in three regions (=oblasts) of KAZ, in East Kazakhstan (EKO) and Akmola (AkO) regions, and Almaty city (AO) that are officially registered as TBE endemic regions (figure 2) in the TBE endemic season from April to October of the years 2018 and 2019. Blood and CSF sampling was conducted in patients with suspected cases of meningitis in eight hospitals. A suspected case of meningitis was defined as an illness with sudden onset of fever and one or more of the following symptoms: neck stiffness, altered consciousness, or other meningeal signs.

Additional inclusion criteria were persistent headache and vomiting. Exclusion criteria were an age below 18 years and mental conditions such as psychosis or uncontrolled depression as these conditions may interfere with the ability to comprehend an informed consent form, and the adequate self-assessment when completing the study questionnaire. Upon inclusion prospective participants were asked to fill in a questionnaire and then two blood samples were taken, one at the

day of hospitalisation and the second one 10-14 days later, and CSF were collected. Blood samples were collected in a vacutainer with a coagulation activator and subsequently centrifuged to separate the serum. All serum samples were transferred into cryotubes and stored, as the CSF, at -20°C until further analysis and/or transportation. The material transporting from East Kazakhstan and Akmola regions to Almaty city (place of the BSL3 laboratory) were organized by local sanitary epidemiological stations with special permission for biological samples transporting according to Order of the Minister of Health of the Republic of Kazakhstan dated September 8, 2017 No. 684. Samples were packed according to the biosafety rules in a triple pack and transported with dry ice with maintenance of the required temperature regime -20ºC. Only patients where the two sera were available were included in the study. A lack of CSF or a not fully completed questionnaire was not considered as an exclusion criterion.

2.2.2 Questionnaire

The pen and paper survey consists of seven modules that included 39 questions on topic such as socio-demographic factors, living and lodging conditions, contact to livestock and vector habitats, a recent tick bites, acute clinical symptoms, and TBE vaccination status information and vaccination history. The questionnaire was self-administered and was supported by hospital personnel. Each patient got an ID number to ensure anonymization and all data was then entered into an Excel (Microsoft Office 15, Redmond, USA) database, which was later imported into Stata (StataCorp, LLC, College Station, USA) for statistical analysis.

2.2.2.1 Description of variables assessed in the questionnaire

First, some general information was obtained by asking the following points In Module 1 Socio-demographic information:

- Interviewer Name: Name of the doctor that performed the questionnaire with the participant.
- Hospital ID number: a hospital Identification code (abbreviation of the city or district) was allocated to each participant.
- Date of Interview in DD.MM.YYYY when Interview was performed with participant.
- Participant ID: Participants were anonymized by only one Participant Identification number**,** in form of a four-digit number, starting at 0001.
- Sex of participant: gender, considered as (1) male and (2) female.
- Name of study site: categorizes the area where study was conducted as (1) Almaty region, (2) East Kazakhstan, (3) North Kazakhstan, (4) West Kazakhstan and (5) Kyzylorda.

Second, Detailed Information about the participant was obtained by the following points:

- When were you born? This was described a date of birth in DD. MM. YYYY. In the case if day and/or month is unknown “99” or year “9999” was entered
- How old are you: gives information about the age of the participant in three units, in case it was not known 999 was entered.
- Where were you born? indicates the country of birth categorized in (1) Kazakhstan, (2) Kirgizstan, (3) Uzbekistan and (4) Other country including a line for specification of it.
- In which oblast of Kazakhstan were you born: indicates in which oblast (administrative areas) the participant was born.
- In which city/town/village do you live: indicates the current living address of a volunteer.
- Since when have you been living in this city/town/village? defines the collective years of living in the city or town or village, categorized as (1) Always lived in this place, (2) Years in three digits. In case if answer was not known marked as “999”.
- Have you done any trips from your place of residence within the last month: indicates the possible journeys and categorized (1) No and (2) Yes, specify where.
- Have you done a trip into nature within the last month: indicated if the participant travelled to nature sites and is categorized into (1) No and (2) Yes, specify where.
- Have you had contact with wild animals: indicates the probable contact with wild animal and categorized as (1) No and (2) Yes, specify where.
- Have you been bitten by ticks, mosquitoes, insects or wild animals within the last month: indicates the possible bites of ticks, mosquitoes or wild animals and categorized as (1) No and (2) Yes, specify what kind of.

Third, detailed information about Living and Housing are gathered in Module 2 in following points:

- What type is your flat/house: categorizes the living standard according to (1) well-equipped city apartment, (2) poorly equipped city apartment, (3) private well-equipped house and (4) poorly equipped house. In case none of them fits other living standards can be named.
- Did you drink raw milk directly from the animal or eat raw milk products without pasteurization: indicates the consumption of non-pasteurized milk products indicated by (1) no and (2) yes and if yes, the type of animal should be specified.

In Module 3: detailed information about contact to livestock should be obtained by following points:

- What kind of livestock does your household own and how many of each species: numbers of owned cattle, horses, goats, sheep, cat/dogs and other animals were indicated in this point. If the real number is unknown “000” was enter for none and “999” for correct numbers were not known.

- How often do you have direct contact to the above mentioned animals: indicates working with these animals, like milking, slaughtering, handling raw meat and excludes the consumption of cooked meat. Contact can be categorized in (1) always/daily, (2) most of the times, (3) rarely and (4) never by considering following species of animals: cattle, horse, goats, sheep and cats/dogs.

Next, Vector habitat factors are identified in Module 4 in the following points:

(4) Where is your house located, in urban or rural area: indicates living surroundings in (1) rural area and (2) urban area.
(5) What kind of ground is around your residence: specifies living surroundings categorized in (1) tarmac, (2) sand and (3) dirt.
(6) How does the vegetation around your residence look like: characterizes the nature surrounding of the living area in (1) dense plantation/ forest, (2) large grass fields, (3) occasional bush agricultural fields, (4) swamp, (5) lake, (6) forest and (7) others, to specify.

Module 5 focuses on Tick bites in the following points:

(7) How many tick bites do you remember during all of your live: indicates the amount of tick bites all of the participants life by (1) none, (2) 1-50, (3) 51-100, (4) 101-200 and (5) more than 200.
(8) How many tick bites do you remember within the last 12 months: indicates the amount of tick bites happening the last 12 months to the participants by (1) none, (2) 1-10, (3) 11-20, (4) 21-50 and (5) more than 50.
(9) How does the vegetation around your residence look like: indicates the surroundings of the housing by (1) dense plantation/forest, (2) larger grass fields, (3) occasional bush agricultural fields, (4) swamp, (5) lake, (6) forest and (7) others to specify.

Clinical symptoms are evaluated in module 6 by following points:

(10) Beginning of the symptoms indicates the onset (1) less than 5 days ago and (2) morethan 5 days ago.
(11) Please indicate below which symptom(s) you have: symptoms are categorized in (1) fever, (2) headache, (3) neck pain (meningism), (4) weakness of muscles or joints, (5) muscle paint or recurrent cramps, (6) pain when swallowing, (7) joint pain, (8) stom- ach/abdominal pain/cramps, (9) back pain, (10) earache, (11) couch, (12) difficulties in speaking, hearing or seeing, (13) seizures/epilepsy, (14) difficulties in breathing,
(15) rapid breathing, (16) sore threat, (17) congestion of nose, (18) enlarged lymph nodes and (19) icterus.

(12) Body temperature: is indicated by ear measurement in (1) less than 37.5°C and (2)more than 37.5°C.

(13) Duration of high body temperature: has to be answered if body temperature is morethan 37.5°C and is categorized in (1) less than 3 days and (2) 3 days and more.

(14) Characterization of blood pressure by (1) normal, (2) hypotension or (3) hypertension.

(15) Characterization of pulse rate by (1) less than 80, (2) 80-100 and (3) more than 100.

(16) Skin conditions: in this point it should be indicated if following symptoms are present: (1) exanthema, (2) ulceration, (3) edema and (4) others.

(17) The Urine is characterized by (1) blood in urine, (2) pain on urinating, (3) dark urine, (4) low urine volume or (5) others to specify.

(18) If any Medications are taken is indicated in (1) antipyretics, (2) antirheumatics, (3)antibodies and (4) others to specify.

(19) Duration of therapy: indicates how long the medications have been taken in days.

(20) Similar illnesses in the family or in the surroundings are indicated by (1) Yes or (2) No.

The status of prophylaxis is indicated in Module 7: Prophylaxis

- Did the participant receive a Vaccination against TBE: (1) No or (2) Yes and if yes specify when.
- Did the participant receive a specific anti-TBEV IgG: (1) No or (2) Yes and if yes specify when.

2.2.3 Sample size

To calculate the sample size for this study, we used the below formular which estimates the prevalence of a parameter within a community or population yet providing a sample size conservative enough to be a representative portion of the population (Pourhoseingholi et al., 2013). The sample size calculation was performed by using the following formula:

$$n = \frac{Z^2 p(1-p)}{d^2} \qquad \mathrm{n} = \frac{1{,}96^2 \times 0{,}1\ (1-0{,}1)}{0{,}05^2} = 139$$

Explanation of variables: n – number of samples required; Z – for CI of 95%, z = 1,96 normal distribution table; p – estimated prevalence based on previous study, d – level of precision.

The above depicted formula constitutes of the Z which is the Z-score and is set at 1.96, p which is the estimated prevalence of flaviviridae infection in Kazakhstan which was estimated at 10% in the individual regions of Kazakhstan and covered population type and d which is the acceptable

type I error margin set at 5%. Using the above formula, we calculated a minimum sample size of 139 participants. To ensure completeness of data for all participants we further added 20% to compensate for the potential non-responders. This led to a minimum sample size of 167 participants for the study.

2.2.4 Serum inactivation

All steps in this procedure were performed in a Class 2 certified biosafety cabinet (BSC) in a biosafety level (BSL) 2 laboratory. The virus becomes inactive when exposed to heat because heating acts on the viral capsid and envelope and causes an structural alteration of viral proteins that disrupts the specific structures necessary to recognize and bind to host cells, thereby inhibiting replication. This can make it impossible to penetrate the cell membrane or cell envelope of the host as well as introducing viral RNA or DNA into the host cell genome. Sera inactivation was carried out in a water bath heated to 56°C for 60 minutes. Before starting the work personal protective equipment such as a gown, and gloves had to be put on, and the laboratory working place and the pipettes were cleaned by antiviral disinfectant and a waste bag for used tips was prepared. The microtubes with the frozen sera were taken out of the freezer in advance so that their contents had time to thaw at room temperature. Then they were centrifuged at 6,000 g/8,000 rpm using a microcentrifuge inside the BSC to prevent contamination through splashing of material from the tube lid when the tube is opened. Subsequently 200 µl of serum sample was transferred into the prepared clean and signed 1.5 ml microtubes with a tight-fitting hinged lid. The tubes with sera were placed in a preheated 56°C water bath for 60 minutes for inactivation. Afterwards the tubes were taken out of the water bath, dried with paper towels and put to racks ready for the following work. The inactivated samples were stored at -20°C.

2.2.5 Analysis pipeline of human sera

All sera samples were investigated by ELISA using commercial kits according to the manufacturers' instructions. The Anti-TBEV IgM/G ELISA kit (Euroimmun, Luebeck, Germany) was used for the TBEV ELISA investigation. For the identification of WNFV by ELISA the Anti-WNFV IgM kit (Vector best, Novosibirsk, Russia) was used, and the Anti-WNFV IgG kit (Euroimmun, Luebeck, Germany) was employed for identification of antibodies in the sera.

First the second serum of a patient was screened for Anti-TBEV IgG antibodies by ELISA. In samples that recurred positive, the first serum was also subsequently checked. In the case that both sera were positive for anti-TBEV IgG antibodies a titration was performed to check if there is a measurable four-fold rise of the antibody titer. The titration results indicate whether the infection with TBEV was in the past or acute.

To determine class M immunoglobulins (IgM) against TBEV, we examined all serum samples, both the first and second. Again, if of both sera were positive in the initial ELISA a titration was performed.

The WNFV investigation was organized in the same way as for TBEV.

After this initial screening for IgM/IgG immunoglobulins by ELISA the first serum from patients that tested positive for TBEV or WNFV antibodies were also checked for anti-Cytomegalovirus (CMV) and anti-Epstein-Barr virus (EBV) IgM with ELISA (Euroimmun, Luebeck, Germany) to exclude respective infections with EBV or CMV that could result in false-positive results in the TBEV or WNFV assays.

All sera reacting positive in the TBEV and WNFV IgM/G ELISA were further tested using an immunofluorescence assay (Euroimmun, Luebeck, Germany). This assay has better specificity as equated to the ELISA assays. So, potential cross-reactivity with antibodies produced by the exposure of patients to other flaviviruses can be excluded.

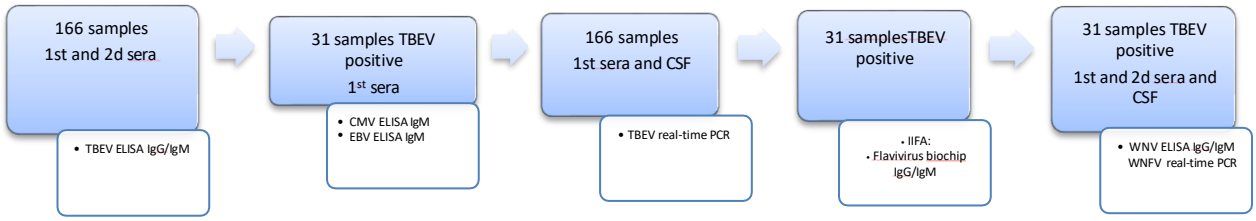

Figure 5: Pipeline of the serological investigation of sera and CSF.

2.2.6 Serum and CSF investigation with ELISA on TBEV IgM/G

Both first and second sera samples were analysed for TBEV-specific IgM/G antibodies using a commercial ELISA kit according to the manufacture instructions (Anti-TBEV IgM/G ELISA; Euroimmun, Luebeck, Germany) using a semi-quantitative method with a standard calibrator and the preparation and dilution process according to the instruction of the manual. The Anti-TBEV IgG kit has included three calibrators that are used for the quantitative method, the Anti-TBEV IgM kit contains only one standard calibrator. In our study for all the ELISA investigations the semi-quantitative method was applied.

All sera samples were diluted 1:100 (10 µl of sera and 1,000 µl of the sample buffer as recommended in manufacture instructions) with the sample buffer in prelabelled tubes 1,5 ml. 100 µl of calibrator, negative, positive controls, and preliminary diluted patients' sera were added into the strip wells included in the kit. Then the samples were incubated for 30 min at room temperature. During the first incubation, the specific IgM of patient sera binds to antigens immobilized on the

surface of wells of strips. Not bound antibodies are removed by washing. After the incubation, the wells were rinsed using the washing solution (Tecan Hydroflex). Each well had to be washed at least with 450 µl of washing solution during the washing cycle. Before use, the 10X washing stock solution had to be diluted with 900 ml of distilled water. The wells were washed 3 times. The time interval between filling and emptying of the wells had to be not less than 30 seconds, best is between 30-60 seconds. After the washing, the strip wells were dried by tapping the upturned plate onto a paper tissue. After washing the conjugate (peroxidase-labelled anti-human IgM antibodies) was added, 100 µl to each well of the plate. After a following incubation for 30 min, at room temperature wells were washed and dried again. Next, wells were incubated with a specific IgM-interacting conjugate (enzyme-labelled anti-human antibodies) which catalyses a colour-shift. The resulting colour intensity is proportional to the amount of TBEV specific IgM antibodies present in the test sample. This was followed by pipetting 100 µl of chromo-gen/substrate into each well with a following incubation of 15 min in a dark place. Into the same wells without washing a stop solution was added. Then the plate with the stained strips were analysed for their colour intensity in a plate reader (Tecan Infinite F50, Country of origin). The filter used for measuring had a wavelength of 450 nm. The evaluation of results was done by calculating the ratio of the control wells to the sera incubated wells. The ratio is formed from the value of the control or a sample divided by the extinction of a calibrator. The result of the analysis was categorised as positive if the ratio was larger or equal to 1.1, negative if the ratio was below 0.8, and undetermined if the ratio was between 0.8 and 1.1. After the ELISA procedure was finished, the working place had to be cleaned.

2.2.7 ELISA WNFV IgM/G investigation

For detecting anti-WNFV IgM antibodies a commercial ELISA kit was used (Vector best ELISA kit) and the preparation of the samples was performed according to the manufacturer's instructions. All sera samples were diluted 1:10 (10 µl of sera and 90 µl of the sample buffer) with the sample buffer in a sample predilution plate. Correct dilution is indicated by a shift of the colour of the sample buffer from crimson to yellow. Further, 100 µl of negative control were put into two wells of a sample strip, into a third well 100 µl of positive control was added. In the remaining wells, 90 µl of serum dilution solution had to be added to the wells topped up with 10 µl of prediluted samples. The plate was sealed with a film and incubated in a thermostat for 60 minutes at a temperature of 37°C. At the end of the incubation, the sticky film was removed and placed in a vessel with a disinfectant solution. Using a washing-module equipped plate reader (Tecan Hydroflex) for washing, the wells were washed 5 times with the wash solution provided by the kit. Each well had to be washed at least with 400 µl of washing solution during the washing cycle. The time between filling and emptying the wells should be at least 30 seconds. The washing solution was

25x concentration and 10 ml had to be diluted with 240 ml of distilled water. After the washing, the strips had to be dried by tapping the upturned plate onto a paper tissue. After drying, the conjugate (polyclonal antibodies to WNFV conjugated with horseradish peroxidase) prediluted with WNFV antigen mix (for 10 strips 10 ml of conjugate were added to 1 ml of WNFV antigen mix solution) was added 100 µl to each well of the plate. The plate was sealed with a film and incubated in a thermostat for 90 minutes at a temperature of 37°C. Subsequently a washing step was performed again. The next step consisted of pipetting 100 µl of chromogen/substrate (tetramethylbenzidine) into each well with a following incubation for 25 min in the dark. After incubation, into the same wells without washing 100 µl of a stop solution were added and the plate was quantified in a plate reader (Tecan Infinite F50, Hombrechtikon, Switzerland). The main filter was selected for 450 nm. The evaluation of results was done by calculating the ratio of samples compared to the controls. The ratio is the arithmetic mean of the optical density in the wells with a negative control sample plus 0.2. The result of the analysis was positive if the ratio was equal or higher than 1.1, negative if the ratio was below 0.8, and indeterminate if the ratio spanned between 0.8 and 1.1.

2.2.8 ELISA EBV and CMV IgM investigation

The investigation of IgM antibodies against EBV and CMV was performed with commercial kits (Euroimmun, Luebeck, Germany) in the same way as it was described above for anti-TBEV IgM.

2.2.9 Immunofluorescence assay

An immunofluorescence assay is a method to detect the existence of reactive antibodies in a serum from a patient or animal. In this commercially available tool, microscope readable biochip slides are precoated with cells infected with various kinds of viruses. After incubation of the slides with the serum of interest the slides are treated with green fluorescence protein (GFP) labelled secondary antibodies that identify the heavy chain of the patients' antibodies that have bound to the virus infected cells. These secondary antibodies can then be visualised in a microscope equipped with a fluorescence light source. To perform such an immunofluorescence assay specific for an array of flaviviruses, samples were first diluted 1:10 and 1:100 according to the kits instructions (IIFA: Flavivirus Mosaic IgM/G, Luebeck, Germany). The slide preparation of the samples was performed according to the manufacturer's instructions. The diluted samples were applied in a volume of 30 µl to each reaction field of the reagent tray, avoiding air bubbles. All samples to be tested should be transfer before starting the incubation. The slides were incubated for 30 minutes at room temperature. Then the biochip slides were rinsed with a flush of PBS-Tween using a beaker and they were immersed in a cuvette that contained PBS-Tween for 5 minutes. The container was shaken for 5 minutes with a rotary shaker. The washing buffer was prepared in advance by dissolving one pack of "Salt for PBS" in one litre of distilled water and mixed with

two millilitres of Tween 20 and stirred for 20 minutes. Then the slides were removed from the cuvette and blotted with a paper towel (only the back side of slides). After this primary antibody incubation 25 µl of the conjugate were applied to each reaction field and incubated for 30 minutes at room temperature in a place protected from light. After the incubation the biochip slides were again rinsed with a flush of PBS-Tween using a beaker and they were immersed in a cuvette that contained fresh PBS-Tween for 5 minutes that was shaken for 5 minutes with a rotary shaker. Afterwards the slides were removed from the washing buffer and air dried. Finally, 10 µl of mounting medium was placed on a cover glass and carefully fixed on the biochip with the mounting medium facing downwards. The prepared biochips were stored at 4°C until analysis. The biochip analysis was performed using a MicroOptix MX 300 fluorescence microscope and a 40x magnification. Slides were screened for cells that expressed an obvious positive GFP signal. The titre of the antibody levels in the patients' serum was derived from this biochip analysis from the sample dilution factor for which specific fluorescence is visible. In this study, samples were checked for IgM and IgG antibodies reactive against four additional flaviviruses, TBEV, WNFV, JEV (Japanese encephalitis virus), and YFV (Yellow Fever virus).

2.2.10 RNA extraction of viral genomes from CSF and sera samples

The RNA extraction was performed for screening for the presence TBEV, WNFV and OHFV RNA in the sera sampled from patients on their day of hospital admission and in CSF. The extraction and performed with the QiAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The RNA extraction can be divided into two steps, first a lysis and then a viral mini spin RNA isolation procedure. First, the sample is lysed under denaturing conditions provided by the AVL buffer supplemented with carrier RNA. The buffer AVL inhibits RNAses and stabilizes viral RNA. At the same time the carrier RNA improves the binding of viral RNA to the QIAamp membrane and reduces the chance of viral RNA degradation. Moreover, during this procedure all active virus particles in the sample are inactivated. After inactivation the tubes with serum or CSF are centrifuged for five minutes at 6,000 g (8,000 rpm). All these steps have to be conducted under the flow hood. In this procedure we used only RNase free tubes. These pre-signed tubes were filled in with 560 µl AVL buffer and 5.6 µl of carrier RNA, then 140 µl of the sample (supernatant fluid) was added. The tubes were vortexed for 15 seconds. After shaking, the tubes were incubated at room temperature for 10 min. During the following procedure the RNA binds to the membrane and contaminants are washed away in two steps using two different washing buffers. These latter steps should be conducted in the special RNA extraction area. The whole procedure requires this special conditions to avoid a contamination and to reduce the chances of RNA degradation as RNAases are ubiquitous. Subsequently 560 µl Ethanol (96-100%) were added to each sample, carefully shaken, and pulse-centrifuged to remove liquid from the top of the tubes. For each sample an individual spin column

in a collection tube was prepared. From the lysed sample 630 µl were added to the spin columns without touching the filter material in the QIAamp Mini Spin Columns and then centrifuged at 6,000 g (8,000 rpm) for one minute. The eluate was discarded. Afterwards 500 µl of AW1 buffer was added on the column without touching the column material, then again centrifuged for one minute at 6,000 g/8,000 rpm and resulting eluate was discarded. Then, 500 µl of AW2 buffer were added into the tubes and centrifuged for three minutes at 20000 g (13000-14000 rpm). Then the collection tubes were changed and Spin Columns were put into new collection tubes and centrifuged at 20,000 g (14,000 rpm). The washed QIAamp Mini Spin Columns were put into new sterile tubes (1.5 ml). To eluate the RNA, 40 µl of AVE buffer was added carefully and then column in the collection tubes were centrifuge for one minute at 6,000 g/8,000 rpm. This step was performed twice to elate all the RNA from the filter in the column. The resulting 80 µl of AVL containing RNA were subsequently aliquoted in 20 µl aliquots and stored in a freezer at -80°C.

2.2.11 Real time RT-PCRs and target E-gene conventional PCR.

To screen for TBEV RNA in the samples two different PCR protocols where applied. First, RNA extracted from serum and CSF was tested by real-time RT-PCR using a Quiagen Rotor Gene Q device (Schwaiger M, 2003) with the QIAGEN QuantiTect Virus Kit for TBEV. Extracted strains of Langat virus (Acc. Number NC_003690) were used as positive control and distilled water served as negative control.

The WNFV was also investigated by real-time RT-PCR on a Rotor-Gene Q machine but the employed chemistry was provided by AmpliSens WNV-FL (Russia) according the instructions of the manufacutrer (Baturin, 2021).

Table 2: List of primers used to screen for TBEV in real time RT PCR

Primer name	Sequence
F-TBE 1	5’-GGGCGGTTCTTGTTCTCC-3’
R-TBE 1	5’-ACACATCACCTCCTTgTCAgACT-3’
hybridization probe	5’-6FAM-TGAGCCACCATCACCCAGACACA-TMR-3’

All samples that responded a positive signal in the first step were rechecked in a second analysis step by a conventional RT-PCR. Here the target of the used primers was the E-gene (1,687 bp) of TBEV.

Table 3: Primer sequences for the E-gene analysis for TBEV in CSF

Primer name		Sequence
RSSE 947A	Forward	5'-TCC TCT GCC TGG CTC CGG TTT ATG-3
RSSE 947B	Forward	5'-TCT TGT GCC TGG CTC CGG TTT ATG-3
RSSEc2579	Reverse	5'-CCT GGC GTT TCT GGG TAG TAT G -3'
TBEw c1648	Alternative reverse primer optimized for sequencing	5'- GCAGAGCCAGATCATTGAACC -3'

The amplification in the conventional RT-PCR was performed in 45 cycles with an annealing temperature of 52°C, catalysed by the Invitrogen™ SuperScript™ III One-Step RT-PCR system. The initial amplification of the cDNA was performed at 50°C for 45 minutes, then a step of denaturation at 94°C for 5 min was carried out. The amplification was conducted for 45 cycles at 94°C for 30 sec, 52-60°C (according to the annealing temperature written in the primer instructions) during 30 s and 2 min at 68°C. A final extension step at 68°C for 8 min was done. The PCR products were visualised on a 1.5% agarose gel stained with Gel Red® under the illumination of ultraviolet light. In a third step, all the samples that gained PCR products in the first and the second PCR run were isolated and set to be sequenced by the Sanger method (sequence termination) with the ABI Prism Big Dye Terminator V3.1 Cycle Sequencing Kit and 3500xl Genetic Analyser machine. The sequencing was initiated by using the initial primers of the RT-PCR amplification (Table 3).

2.3 Tick molecular prevalence study

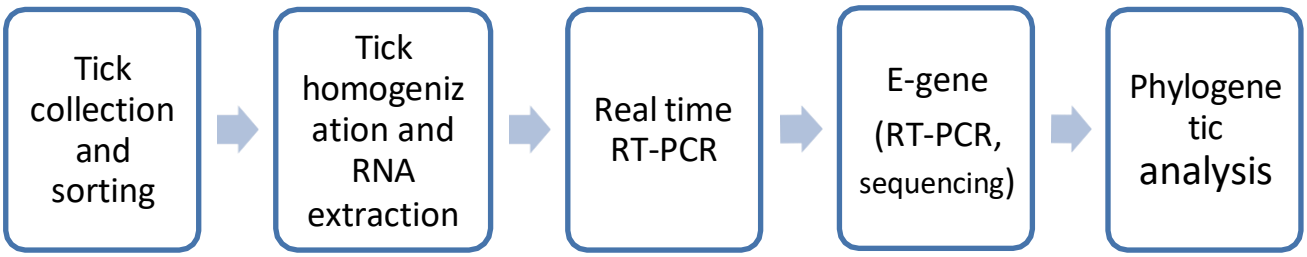

Figure 6: Tick investigation scheme

2.3.1 Tick collection

Ticks were collected by flagging in the three selected regions Akmola oblast, East Kazakhstan oblast and Almaty oblast from March to June, during the peak activity of ticks, in the years of 2016-2019. Exact sampling areas were chosen according to the standard routes of tick collection by workers of the Scientific Practical Center for Epidemiological Expertise and Monitoring (SPCEEM). For each sampling site exact GPS data were noted.

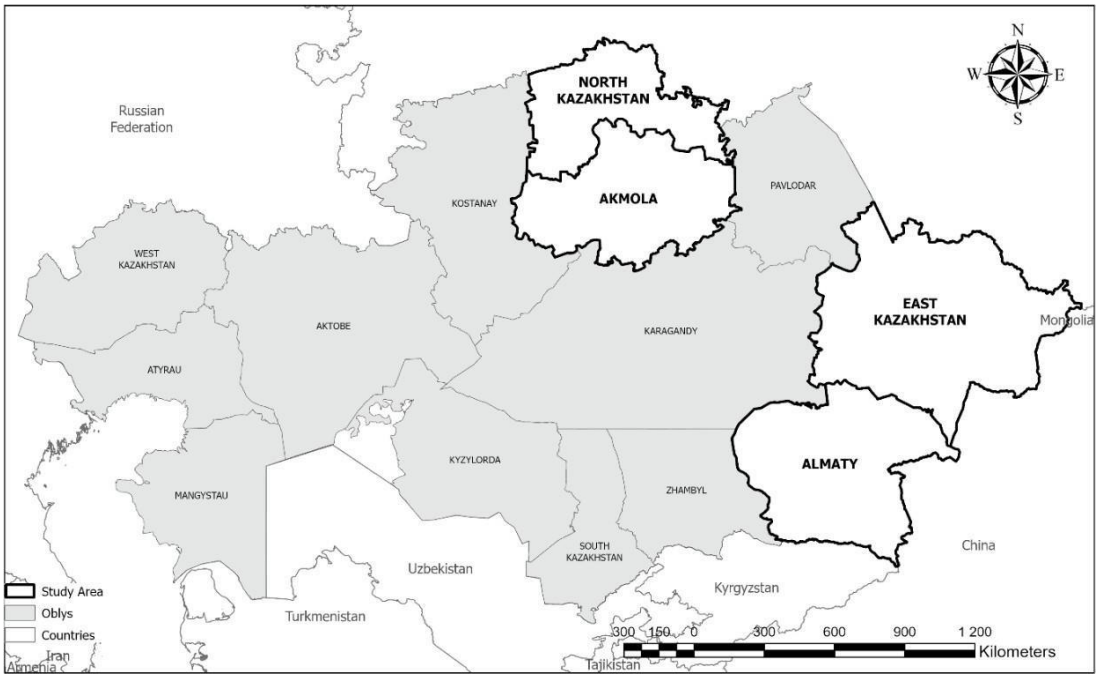

Figure 7: Map of Kazakhstan showing the four regions where the tick collection was performed.

2.3.2 Tick sorting

The collected ticks were morphologically identified and sorted by species, sex, gender and stage of life (table 4). Collected ticks (n=10,038) were sorted into pools of five ticks in the BSL 2 laboratory of the SPCEEM in Almaty city. Handling of the ticks was with the support of an experienced entomologist. Each pool contained five imago ticks of the same species; congested ticks were sorted one by one.

2.3.3 Tick homogenization

Tick homogenization was performed in a BSL 3 laboratory of NSCEDI, because of the risk of presence of especially dangerous infections in the ticks such as Crimean Congo haemorrhagic fever that is known to reside in neighbouring regions to the areas of interest. Two 4 mm metal (stainless steel) balls were put into a clean 2 ml tube with a tight lid and 1,000 µl DMEM were added to each of these test tubes in a BSC-II. All tubes were labelled in advance and then loaded

with the pool of five identical ticks. The test tubes were placed into a tissue lyser (TissueLyzer, Qiagen, Germany) between two cooled blocks and ticks were homogenized with a rigorous shaking for 2 min at a frequency of 30 Hz. After homogenization all tubes were centrifuged 15 minutes at 6,000 g/8,000 rpm to sediment the homogenate and supernatant was transferred into a fresh tube and stored at -80°C until further use.

2.3.4 RNA Extraction and real time RT PCR

The RNA extraction and screening with real time PCR were organized the same as it was described in the part on serological investigation.

2.3.5 Minimum infection rate calculation

To calculate the prevalence of tick-borne encephalitis virus in ticks, the minimum infection rate (MIR) was used, given the assumption that not more than one tick from each pool was positive (Gu et al., 2008). As flaviviruses show a low prevalence, less than 5%, the best way to calculate the prevalence of the virus is to use the MIR. The MIR was calculated for each sampling site.

2.3.6 Amplification of the TBEV E-gene with conventional RT-PCR

The E-gene was amplified from positive samples of the real-time RT PCR using a conventional RT PCR with primers targeting the E gene fragment (1,632 bp) as it was mentioned before. Different combinations of reverse and forward primers were used (Frey et al., 2012).

Table 4: E-gene primer pairs for TBEV sequencing in ticks

Primer name	direction	Sequence
RSSE 947 A + RSSE 947 B	Forward	5'-TCCTCTGCCTGGCTCCGGTTTATG -3'
		5'- TCT TGT GCC TGG CTC CGG TTT ATG -3'
RSSEc2579	Reverse	5'- CCT GGC GTTTCT GGG TAG TAT G -3'
TBEw885	Forward	5'-GGTTACCGTTGTGTGGTTGACC-3'
RSSEc2579	Reverse	5'- CCT GGC GTTTCT GGG TAG TAT G -3'
RSSE952	Forward	5'- TGC CTG GCT CCG GTT TAT GC -3'
RSSEc2579	reverse	5'- CCT GGC GTTTCT GGG TAG TAT G -3'

TBEw c1648:	Alternative reverse primer optimized for sequencing	5'- GCAGAGCCAGATCATTGAACC -3'

The amplification produces a segment of 1,632 bp length. In the subsequent phylogenetic analysis from both ends parts of the sequence and the primers are removed, so the final sequence length that is used for further phylogenetic analysis for the E-gene is about 1,422 bp. As the E-gene fragment is too long and during the sequencing the quality of such a long sample is declining a transitionary alternative reverse primer optimized for sequencing was used (TBEw c1648) to yield a better quality of sequencing.

3. Results

3.1 Serological investigation of human blood and CSF samples

Altogether 179 patients with supposed cases of meningitis from eight hospitals located in three regions of KAZ were enrolled in this study.

Table 5: Overview of all samples from patients with alleged sympotoms of meningitis or meningoencephalitis from three regions of Kazakhstan.

	Collected samples		
region/hospital	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**

Nevertheless, thirteen sera samples had to be excluded from the study since a second serum was not collected. These were six patients from Almaty, six samples from East Kazakhstan and one sample from Akmola region. Therefore, the total number of patients included in this study is 166. Furthermore, from 130 of this 166 patients CSF was collected.

The information collected from the questionnaires amounted to 179 samples, these are the data that will be given below, and the number of samples investigated was 166, since 13 were excluded from the study due to absence of a second serum.

The most frequent symptoms in patients with serous meningitis of unknown aetiology as reported in the questionnaire were headache (n=171/178, 96.1%), fever (n=145/178, 81.5%), and neck

pain (n=80/178, 44.9%) (Table 6). The frequency of other symptoms was low and mostly depended from underlying medical conditions.

Table 6: Clinical symptoms recorded among patients with suspected cases of meningitis in three regions of Kazakhstan.

Symptom	**TBE** (% of all enrolled patients that presented the symptom)	**WNF** (% of all enrolled patients that presented the symptom)	**Flaviviridae** (% of all enrolled patients that presented the symptom)	**Negative for Flaviviridae** (% of all enrolled patients that presented the)	**Total** (% of all enrolled patients) 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)

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

From all enrolled patients, only one patient got a vaccination against TBEV in July 2019 and additionally received an anti-TBEV specific IgG injection as post-exposure prophylaxis after a tick bite in September 2019. None of the patients were vaccinated against other flaviviruses such as Japanese Encephalitis Virus or Yellow Fever Virus.

The biggest share of collected samples from patients was provided from Almaty city (85.5%, n=153). Other samples were originating from East Kazakhstan (8.9%, n=16) and in Akmola (5.6%, n=10). It is important to mention that the population of only Almaty city is about three million people, and the population of Akmola oblast is 715,000 people, East Kazakhstan oblast – 1.4 million people. Additionally, in spring of 2018 in Almaty city was a registered outbreak of meningococcus infections. The onset of meningococcus meningitis can be presented as serous meningitis and several days later the CSF can show the characteristics of bacterial meningitis.

Among the collected samples, about 60.9% (n=109) of the participants were male. The median age of the patients was 25 years (IQR: 20-32 years) years with the youngest patient being 18 years old and the oldest 81 years old.

The hospitals performed routine laboratory tests, including bacteriological methods conducted in different types of samples, as well as agent specific PCR assays. In 50 patients the diagnoses were confirmed by laboratory methods. In detail, for 29 patients (16.2%) 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 (HIV) in their serum (2.2%), for two (1.1%) patients an infection with borrelia was confirmed, 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*. *Enterovirus* was confirmed by PCR from faeces, *N. meningitidis*, *Streptococcus pneumoniae*, *Staphylococcus sp.*, and *Mycobacterium tuberculosis* were found in CSF by bacteriological method, borreliosis and mumps were confirmed with ELISA in sera. Two patients with HIV, one with cysticercosis and one with borreliosis originated from East Kazakhstan, all other confirmed diagnoses were from Almaty.

In relation to the TBEV endemic season about 65% of patients were screened for TBEV after admission to the hospitals with a Vector Best IgM/IgG ELISA. For nine samples an infection with TBEV was diagnosed as the IgM ELISA was positive, but three of them were excluded because did not had a second sera.

For 63.6% (n=49/77) of the patients a previous trip into the nature was recorded and a tick bite was noticed in 7.8% (n=6/77) of the patients. In 4% (n=7/177) of cases, patients confirmed the use of unpasteurized milk/milk products.

Table 7: Socio-demographic factors and exposure history of patients with alleged symptoms of serous meningitis

	TBEV (% of all enrolled patients that belong to each subgroup)	**WNFV (% of all enrolled patients that belong to each subgroup)**	**Flaviviridae (% of all enrolled patients that belong to each subgroup)**	**Non Flaviviridae (% of all enrolled patients that belong to each subgroup)**	**Total**
Age					
≤ 20	2 (4.3)	0 (0)	2 (4.3)	44 (95.7)	46 (25.7)
21-30	4 (4.8)	0 (0)	4 (4.8)	79 (95.2)	83 (46.4)
31-40	1 (3.2)	2 (6.5)	3 (9.7)	28 (90.3)	31 (17.3)
41-50	1 (10)	0 (0)	1 (10)	9 (90)	10 (5.6)
> 50	2 (22.2)	1 (11.1)	3 (33.3)	6 (66.7)	9 (5.0)
Sex					
Male	10 (9.2)	1 (0.9)	11 (10.1)	98 (89.9)	109 (60.9)
Female	0 (0)	2 (2.9)	2 (2.9)	68 (97.1)	70 (39.1)
Region					
Almaty	4 (2.6)	3 (2.0)	7 (4.6)	145 (95.4)	152 (84.9)
EastKaz	5 (31.3)	0 (0)	5 (31.3)	11 (68.8)	16 (8.9)
Akmola	1 (12.5)	0 (0)	1 (12.5)	7 (87.5)	8 (4.5)
Other	0 (0)	0 (0)	0 (0)	100 (100)	3 (1.7)
Total	10 (5.6)	3 (1.7)	13 (7.3)	166 (92.7)	179 (100)

Trip into Nature (NA n= 102)					
none	0 (0)	1 (3.6)	1 (3.6)	27 (96.4)	28 (36.4)
mountain	0 (0)	0 (0)	0 (0)	19 (100)	19 (24.7)
River/lake	0 (0)	0 (0)	0 (0)	16 (100)	16 (20.8)
field	0 (0)	0 (0)	0 (0)	1 (100)	1 (1.3)
dacha	0 (0)	0 (0)	0 (0)	1 (100)	1 (1.3)
forest	2 (16.7)	0 (0)	2 (16.7)	10 (83.3)	12 (15.6)
Total	2 (2.6)	1 (1.3)	3 (3.9)	74 (96.1)	77 (100)
Types of bites during trip					
Tick bite					
No	1 (1.4)	1 (1.4)	-	69 (97.2)	71 (92.2)
Yes	1 (16.7)	0 (0)	-	5 (83.3)	6 (7.8)
Mosquito bite					
No	2 (2.7)	1 (1.3)	-	72 (96)	75 (97.4)
Yes	0 ()	0 (0)	-	2 (100)	2 (2.6)
Rodent bite					
No	2 (2.6)	1 (1.3)	-	74 (100)	77 (100)
Yes	0 (0)	0 (0)	-	0 (0)	0 (0)
Consumption of raw milk/milk products (NA n=2)					
No	9 (5.3)	3 (1.8)	12 (7.1)	158 (92.9)	170 (96)
Yes	1 (14.3)	0 (0)	1 (14.3)	6 (85.7)	7 (4)
Urban/rural					
Urban	7 (4.5)	3 (1.9)	10 (6.4)	146 (93.6)	156 (87.2)
Rural	3 (13)	0 (0)	3 (13)	20 (87)	23 (12.8)
Vegetation					

Dense plantation	3 (3.2)	1 (1.1)	4 (4.2)	91 (95.8)	95 (54)
Large grass field	2 (12.5)	0 (0)	2 (12.5)	14 (87.5)	16 (9.1)
Agricultural fields	0 (0)	0 (0)	0 (0)	7 (100)	7 (4)
Swamp	2 (66.7)	0 (0)	2 (66.7)	1 (33.3)	3 (1.7)
Lake	0 (0)	1 (100)	1 (100)	0 (0)	1 (0.6)
Forrest with grass field	0 (0)	0 (0)	0 (0)	1 (100)	1 (0.6)
Mixed	3 (5.7)	1 (1.9)	4 (7.5)	49 (92.5)	53 (30.1)
Frequency of tick bites estimated in life					
Never	1 (1.4)	1 (1.4)	8 (4.8)	159 (95.2)	167 (93.3)
1-10	1 (16.7)	0 (0)	4 (40)	6 (60)	10 (5.6)
11-20	0 (0)	0 (0)	0 (0)	1 (100)	1 (0.6)
21-50	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
>50	1 (100)	0 (0)	1 (100)	0 (0)	1 (0.6)

The predominant part of patients either lived in an urban area (87.2%, n=156/179) or resided in an area with dense vegetation (54%, n=95/176). About 9.1% (n=16/176) of the enrolled patients reported to live in the neighbourhood to grassland.

Typical factors correlated to an infection with Flaviviridae 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. All this risk factors were included in the questionnaire. When adjusted in a multivariate model, it exposes that recurrent tick bites significantly increase the probability of getting a flavivirus infection ($p<0.005$). Two further factors, though without any significance when adjusted for other covariates but worth taking note of in the univariate analysis, are age of the patient ($p=0.03$) and the region of residence ($p=0.022$). Older age resulted in higher probabilities of a flavivirus confirmation. Also, a patient

had a higher potential of getting a flavivirus infection when living in East Kazakhstan, Akmola, Russia, or “Other Regions” as compared to a residence in the region around Almaty.

19 out of the 166 samples were positive for IgM antibodies (11.4%) either in the first and/or second serum (Table 8).

Table 8: Seroprevalence of IgG and IgM antibodies against flaviviruses in patients with alleged symptoms of meningitis or meningoencephalitis (ELISA screen).

Region	TBEV IgM ELISA positive (% of positive samples in each region)	TBEV IgG ELISA positive (%of positive samples in each region)	WNF IgM ELISA positive (%of positive samples in each region)	WNF IgG ELISA positive (%of positive sam-ples in each region)
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)

*one patient received TBEV specific IgG for treatment and emergency prophylaxis. % are given as per region.

Table 9: Seroprevalence of IgG and IgM antibodies against flaviviruses (IIFA screen).

	IIFA IgM (1:10, 1:100) (%)				IIFA 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 (2.0)	3 (2.0)	0	6 (4.0)
East (n=10)	3 (30.0)	0	0	3 (30.0)	3* (30.0)	1*(10.0)	1* (10.0)	5 (50.0)
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)

*one patient received TBEV specific IgG for treatment and emergency prophylaxis.

! 2 samples could not be confirmed by IFA

From all IgM positive samples 4.8% (eight out of 166) were positive for TBEV IgM in the first and second sample of the paired sera. A following titration of the sera to guess the antibody content in these eight sera returned comparable titres between first and second serum.

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

In summary in 31 from the 166 patients (18.7%) either IgM or IgG antibodies reactive for TBEV were detected using ELISA as a diagnostic method. Nine patients (5.4%) had IgM antibodies exclusively 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 9). TBEV IgG was confirmed in six (3.6%) specimen and IgM in ten (6.02%) samples by the IIFA (used serum dilutions were 1:10 and 1:100) (Table 9). Five samples had both types of immunoglobulins, IgM and IgG (Figure 9).

Furthermore, WNFV IgM antibodies were revealed in six (3.6%) of the tested IIFA samples and WNFV IgG in four (2.4%) of the samples (Figure 9). Two samples had both, IgM and IgG WNFV antibodies reacting on the flavivirus biochip.

One serum pair from a patient (OSK 7) with an alleged case of meningitis reacted with four viruses blotted on the IIFA biochip (Table 10). Beside a strong signal on the TBEV and WNFV section of the biochip, this patient was also responsive for Yellow fever virus (YFV) and Japanese Encephalitis Virus (JEV) in serum dilutions from 1:10 till 1:400 (Figure 8).

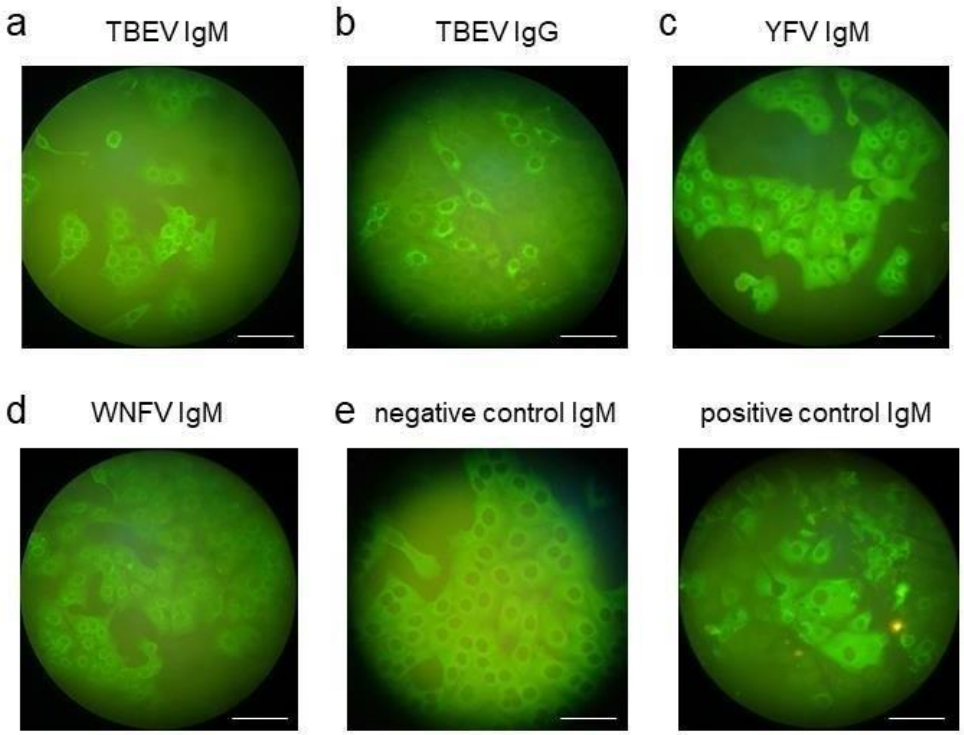

Figure 8: Representative images of the indirect immune fluorescence assay (IIFA). (a) TBEV IgM, (b) TBEV IgG, (c) YFV IgM and (d) WNFV IgM. (e) negative control. Size bar = 50 µm

A further screening for other viruses exposed that one TBEV antibody positive sample also reacted positive for EBV IgM and two samples were reactive in the CMV IgM ELISA. Indeed, one patient (ALM 28) was positive for TBEV IgM, WNFV IgM, EBV IgM and CMV IgM, that represents four acute infections at the same time.

Beside the screen for reactive antibodies in the serum of patients it is also of interest if it is possible to detect viral RNA in the CFS of acute patients by RT-PCR. In this study from the 166 patients a total of 130 CSF specimen were available. For the other 36 patients CSF samples were missing due to difficulties performing the spinal puncture. By screening all CFS samples using TBEV primers, two CSF samples (1.5%) were positive in the TBEV real-time RT-PCR. However, this result could not have been complete using a conventional RT-PCR targeting the E-gene of TBEV and thus no sequences of the E-gene could be generated. Nonetheless, these two CSF samples from patients with suspected patients of meningitis were reactive for TBEV specific antibodies in the ELISA screen for an acute TBEV infection.

To sum this first part of the study, in the south-east region of Kazakhstan, Almaty region, we found six sera from patients containing antibodies against TBEV (Table 10). Two of them had acute TBEV infections, one serum had the characteristics of a previous TBEV infection. This is complemented by two sera with an acute WNFV infection and one previous WNFV infection. In the East

Kazakhstan region, some 2,000 km western of Almaty region, three positive samples were detected resulting in two TBEV acute infections and one historical TBEV infection. In the Akmola region, the region surrounding the capital of Kazakhstan, Nur-Sultan, was one reactive serum pair indicating an acute TBE infection. All seven TBEV positive patients, either with a previous or acute infection were male. Among the WNFV patients two of the three patients were female. A tick bite was registered in three cases. None of the ten patients was vaccinated against TBEV, but one got an anti-TBEV specific IgG injection after the observed tick bite.

Table 10: Overview of 10 ELISA TBEV and WNFV positive samples that were confirmed by IIFA/PCR.

	TBEV ELISA serum		TBEV IIFA serum		WNFV ELISA Serum		WNFV IIFA serum		CSF PCR	Tick bite	AntiTBE Ig*	Hospital diagnosis	Final diagnosis
Sample ID	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG					
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: TBE tick-borne encephalitis, TBEV tick-borne encephalitis virus; WNFV West Nile fever virus, MUO meningitis of unknown origin, CSF – cerebrospinal fluid, ALM Almaty city, GLU Glubokovskiy district, KOK Kokshetau city, OSK Oskemen city

*AntiTBE Ig – human immunoglobulin against tick-borne encephalitis (titer of hemagglutinating antibodies to tick-borne encephalitis virus not less than 1:80), use for TBE treatment and emergency prophylaxis

3.2 Investigation of ticks for their infection rate with TBEV in four regions of Kazakhstan

3.2.1 Tick collection and sorting

During four years (2016-2019) 10,038 ticks in 2,455 pools were collected in four regions of the Republic of Kazakhstan. Sample collection was performed in regions where cases of TBE were registered. As was mentioned before AkO, EKO, and AO are officially endemic regions and in North Kazakhstan oblast where single reported cases of TBE since 2018In Almaty the tick collection was performed in the area where people were bitten by ticks and got TBE, as was reported previously (Shin A., Master thesis, 2017). In details, the tick collection in Almaty can be divided into two parts – in Almaty city and surroundings and the village of Talgar that is located on the northern slopes of the Zailiyskiy Alatau, 25 km East of Almaty. So, in two districts at 15 sites 1,611 ticks were collected in 344 pools. The biggest part of ticks was collected in East Kazakhstan oblast in 2016-2019, totally were collected 4,820 ticks in 1344 pools. The collection was organized in 10 districts at 58 sites.

- District 1 – Ridder city, located in the Ore Altai in a mountain basin at the foot of the Ivanovsky Range, in the upper reaches of the Ulba River (a tributary of the Irtysh). The city is the terminal point of the European route E40 and the extreme eastern branch of the Kazakhstani railways (Leninogorsk station).
- District 2 – Ulan district, a district in the centre of the East Kazakhstan Oblast. The territory of the area is located at the foothill part of the Kalba Mountains, and the entire area is characterized by mountainous terrain, which, depending on the absolute altitude, can be divided into two parts: the middle and low mountains. The area occupied by the middle and low mountains is mainly represented by pastures.
- District 3 – Ust-Kamenogorsk city is the largest city in the east of Kazakhstan and the administrative centre of East Kazakhstan oblast. Ust-Kamenogorsk is located in the eastern part of modern Kazakhstan, at the inflow into the Irtysh River of the Ulba River, approximately 280 km west of Mount Belukha, the highest point of the Altai Mountains and 947 km from the capital Nur-Sultan.
- District 4 – Shemonaiha district located on the Uba River (right tributary of the Irtysh River), 130 km north-west of Ust-Kamenogorsk, towards the border with the Russian Federation (with the Altai Krai).
- District 5 – Zaysan district occupies the south-eastern part of the East Kazakhstan oblast, less than half of the area is mountainous. According to climatic conditions, the area belongs to the dry desert-steppe and alpine tundra-meadow zones.

- District 6 - Glubokovskiy district is located in the North-East of the region. Two-thirds of the area is mountain-taiga terrain. The climate is continental. The Irtysh River and its tributaries Ulba, Uba and others run through the area. The landscape is represented by steppe and mixed coniferous forests.
- District 7 – Kokpekty district is located in the central part of the East Kazakhstan oblast. The relief of area territory of the district is mainly low-lying, in the north - mountain, in the south - northern part of a hollow of lake Zaisan.
- District 8 – Katon-Karagay district district occupies the north-eastern part of the region. It is the extreme eastern point of the country. In the North and North-East the district borders with the Republic of Altai of the Russian Federation, in the south-east with Xinjiang Uygur autonomous district of China. The relief of the area is mountainous, foothill and alpine zones with sharply continental climate. The Katon-Karagaisky State National Nature Park is located in the area.
- District 9 - is a district in the South-East of East Kazakhstan oblast. The district borders Xinjiang Uygur Autonomous Region of China to the south and east. Less than half of the area is mountainous. According to climatic conditions, the area belongs to the dry desert-steppe and alpine tundra-meadow zones.
- District 10 – Semey (Semipalatinsk) city is located in the Western part of East Kazakhstan oblast and is the second largest city in the oblast. At least 456 nuclear tests were carried out at the Semipalatinsk nuclear test site between 1949 and 1989.

In Akmola oblast the tick collection was organized during three years in 2016, 2018 and 2019, totally were collected 2,291 ticks in 488 pools, in 3 districts at 4 sites. Most of TBE cases in AkO were registered near the Sandyktau village that is why the biggest part of ticks was collected around this district.

- District 1 – Sandyktau district is located in the North-Western part of Akmola oblast, where it shares a border with North Kazakhstan oblast. The area of the district is a plain pierced from North to South by parallel ridges.
- District 2 – Zerendy district is located in the North of Akmola oblast. To the West, North and North-East this district borders on North Kazakhstan oblast. The relief is a low-mountainous, shallow-sloping plain.
- District 3 – Burabay district located in the North of the Akmola oblast. It borders to the North on the North Kazakhstan oblast.

The Ministry of Health of the Republic of Kazakhstan officially requested the tick investigation of the North Kazakhstan region, so it was included in this study. Totally tick collection was performed

in five districts at nine sites, during two years 2018 and 2019, with 1316 ticks collected in 279 pools.

- District 1 - Ayrtau district is located on the border of Akmola and North Kazakhstan oblasts, one of the biggest districts of the North.
- District 2 – Musrepov district is the part of North Kazakhstan region, here were registered first cases of TBE in 2018.
- District 3 Petropavlovsk city is the most Northern centre of Kazakhstan, located, 40 km south of the border with Russia. Petropavlovsk is located in the southwestern part of the West Siberian Plain, on the right bank of the Ishim River, the longest tributary of the Irtysh.
- District 4 – Kyzylzharsky district is located in the north of North Kazakhstan oblast. It borders with Tyumen region of the Russian Federation. The district is located along the Ishim River. The relief of the area is dotted with numerous lakes.
- District 5 - Zhumabayev district is bordered to the North and East by the Tyumen and Omsk regions of Russia respectively.

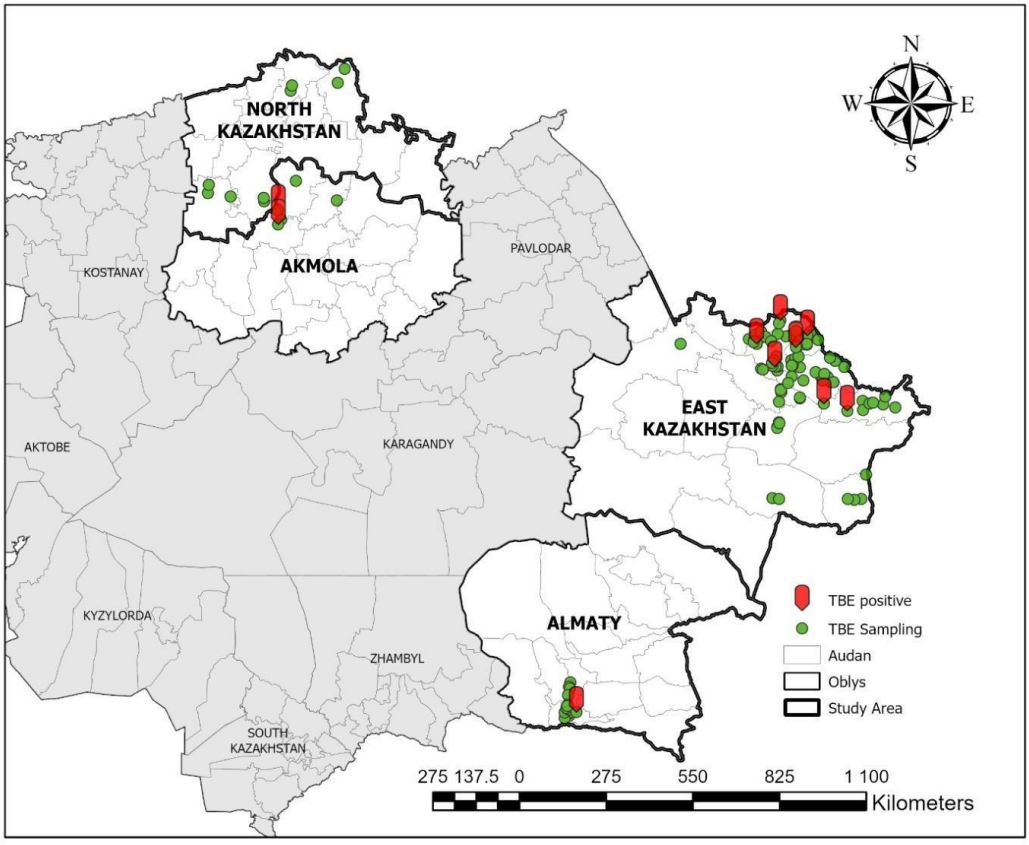

Figure 9: The sites of tick collection in four regions, according to the GPS coordinates.

Among the collected ticks *Dermacentor marginatus* (60.59%) were predominant, other species were presented by *Hyalomma asyaticum* (2.8%), *D. niveus* (0.3%), *Ixodes persulcatus* (12.7%) and *D. pictus* (23.2%), Rhipicephalus *turanicus* (0.01%), *Haemophysalis punctata* (0.6%) (table 4). All ticks presented as imago species.

Table 11: Ticks collected and sorted in four oblasts of Kazakhstan in 2016-2019.

		D. marginatus		*H. asiaticum*	*D. pictus*	*D. niveus*	*R. turanicus*	*H. punctata*	
		East Kasakstan							Pools/ticks
2016		102/353	294/911	71/230	10/14	2/4	2/2	4/4	485/1522
2017		33/104	259/822		29/82			12/58	333/1066
2018		101/432	171/737	9/30	7/29				288/1228
2019		38/143	189/836	2/2	8/22			1/1	238/1004
		Akmola							
2016		2/10	81/400		2/6	5/21			90/437
2018		4/8	9/43		115/550				128/601
2019		6/13	188/904		76/336				270/1253
		Almaty							
2019		5/7	285/1356	5/17					295/1380
		Talgar							
2019		42/205	6/25	1/1					49/231
		North Kazakhstan							
2018		2/2	2/6		74/358				78/366
2019		2/2	9/29		190/919				201/950
Total		337/1279	1493/6069	88/280	511/2316	7/25	2/2	17/63	2,455/10,038

Ixodes persulcatus were of the greatest interest since they are the main vectors of TBEV. The largest share of them were found in Talgar, Almaty oblast, and in East Kazakhstan. In the North Kazakhstan and in Akmola oblast were found only few.

Of all the tested tick pool, 13 out of 2455 (0.52%) pools were positive. Most of the positive samples were from EKO (n=9 out of 13, 69.2%), from AkO two samples turned out positive (n=2 out of 13 15.4%), also from Talgar (Almaty region) two samples (n=2 out of 13, 15.4%) were identified harbouring flavivirus RNA. None of the ticks that were collected in Almaty city were positive (see table 11).

Table 12: Details of tick positive samples.

Sample	Year of collection	Oblast	Region	Species	Sex
EK_8	2016	East Kaz	Ridder city, area of Gromatushinsk canyon	*I.persulcatus*	male
EK_12	2016	East Kaz	Ridder city, area of Gromatushinsk canyon	*I.persulcatus*	female
EK_224	2016	East Kaz	Glubokovo district, area of the village "Gorno-Ulbinka"	*I.persulcatus*	female
EK_347	2016	East Kaz	Katon-Karagay district, area of the village "Mayemer"	*D.marginatus*	male
EK_385	2016	East Kaz	Zyryanov district, area of the village "Maleyevsk"	*I.persulcatus*	female
Akm_44	2016	Akmola	Sandyktau	*I.persulcatus*	female
EK17_164	2017	East Kaz	Glubokovsky district area of the village Predgorny	*D.marginatus*	female
EK18_170	2018	East Kaz	Glubokovsky district area of the village Karaguzhiha	*I.persulcatus*	female
EK18_222	2018	East Kaz	Ridder city, Perviy rayon	*I.persulcatus*	female
TAL_6	2019	Almaty	Talgar	*I.persulcatus*	female
TAL_37	2019	Almaty	Talgar	*I.persulcatus*	female
EK19_24	2019	East Kaz	Ridder city, area of Gromatushinsk canyon	*I.persulcatus*	female
Akm19_37	2019	Akmola	Sandyktau, village Novonilolskoye	*D.marginatus*	female

The screening of tick samples by using TBEV RT-PCR showed MIR in

- Akmola oblast of *I. persulcatus* at village Sandyktau in 2016: 0.002, *D. marginatus* at village Novonikolskoye 0.002.
- Almaty oblast of *I. persulcatus* at Talgar (Zhakynsay mountain) in 2019: 0.008.

- East Kazakhstan oblast in 2016 of *I. persulcatus* at Ridder city, area of Gromatushinsk canyon 0.02, at Glubokovo district, area of the village "Gorno-Ulbinka" 0.14; of *D. marginatus* at Zyryanov district, area of the village "Maleyevsk" 0.03;
- in 2017 of *D. marginatus at* Glubokovsky district area of the village “Predgorny” 0.02;
- in 2018 of *I. persulcatus* at Glubokovsky district area of the village “Karaguzhiha” 0.02; at Ridder city, area of “Perviy rayon” 0.01;
- in 2019 of *I. persulcatus* at Ridder city, area of Gromatushinsk canyon 0.03.

3.2.2 Sequencing of the identified TBEV specimen

Further, all 13 TBEV positive samples were tested with TBEV specific conventional RT PCR with primers targeting the complete E gene, then visualized in a 1.5% agarose gel and subsequently sequenced. Unfortunately, only four samples out of 13 successfully got the full E-gene (1,422 nt) and three more partially. All samples belong to Siberian subtype. The sample of Akmola region (AKM_44) from Sandyktau district belongs to Western Siberia lineage. Samples from Talgar have relationship to Baltic lineage. The found viruses isolated from ticks from East Kazakhstan region are relating to samples from China.

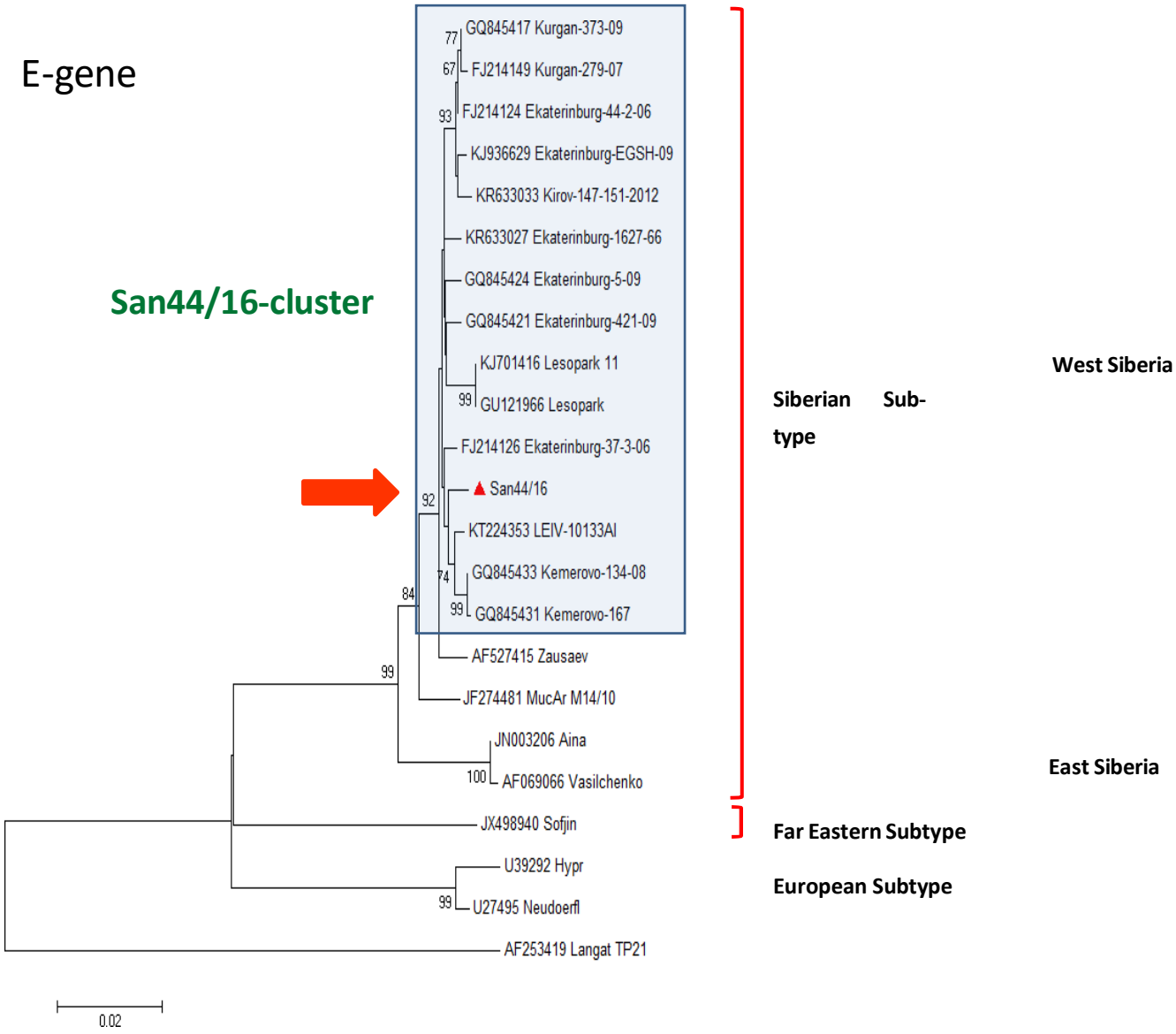

Figure 10: Phylogenetic tree of sample from Akmola (Akm 44), collected in 2016 in the Sandyktau district.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 5). This analysis involved 57 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total

of 1412 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Tamura et al., 2011).

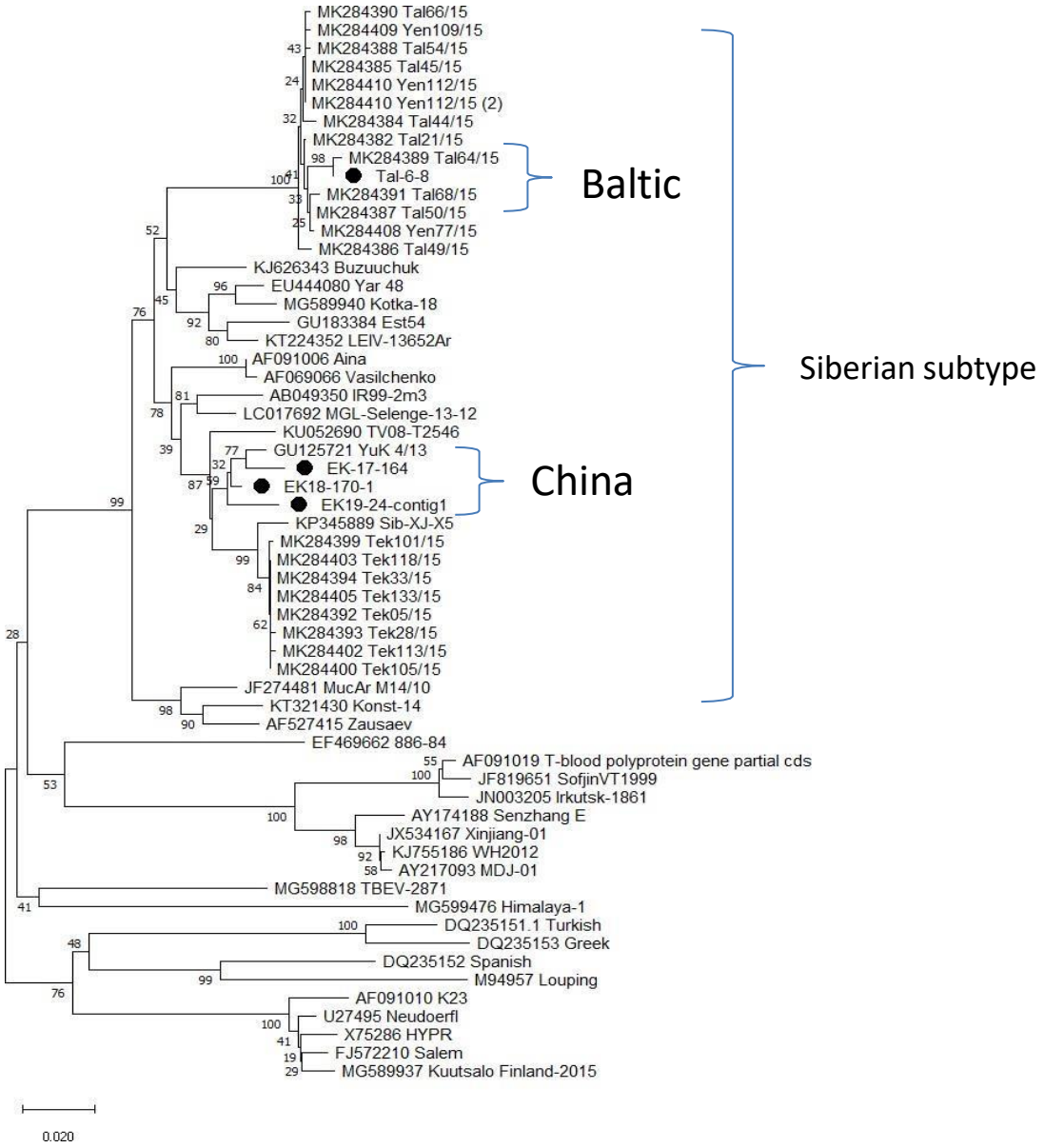

Figure 111: Phylogenetic tree of sample from Talgar (TAL_6) and East Kazakhstan (EK17_164, EK18_170, EK 19_24), collected in 2017-2019

4. Discussion

Frequently meningitis of unknown origin appears in patients and doctors struggle to find a cause of the deteriorating health conditions of patients in their ward. Serous meningitis are often caused by viral infections (Whitley and Gnann, 2002). During the replication the interfering viral particles and incomplete virions that not containing a nucleoid go out from infected cell (Ammosov, 2006). This activates the immune system, induces inflammation and fever. Depending on the type of the virus various organs can be affected, some viruses even pass the blood brain barrier and are able to induce meningitis or meningoencephalitis.

Such an infection can be caused by the Tick-borne encephalitis virus, a virus that is member of the flavivirus family. TBE is widely spread in Eurasia, with single reported cases also in North America. In the Republic of Kazakhstan, a Central Asian country, TBE cases are known and in three oblasts it is officially endemic. All clinical confirmed cases get registered in an annual bulletin.

However, the information about the spread of TBEV in KAZ is incomplete. This is mostly due to an inefficient and incomplete bio surveillance system (Peintner et al., 2021). Furthermore, infectious disease hospitals are only able to perform TBE screenings in endemic regions of the country. In non-endemic areas, doctors have no access to even rudimentary diagnostic tools to check for a flavivirus infection.

TBEV is mostly transmitted via tick bites. However, in Kazakhstan, no screening for TBEV infected ticks is undertaken. But there are efforts to count the numbers of ticks each year in epizoonotical surveillance approaches. Based on this tick numbers, the contamination with TBEV is assessed with mathematical tools. Afterwards, in all the TBEV endemic areas all ticks get chemically removed by the intense spraying of insecticides in areas that are used for recreative activities by the human population.

Only in individual cases, ticks get screened for TBEV antigen. If a person in an endemic region suffers from a tick bite it is a free public health service to bring the tick to the local sanitary epidemiological station, a public health facility, and have it there checked specifically for TBEV. If this tick turns out to be a carrier of TBEV, the affected human is eligible to receive an TBEV prophylaxis, a treatment that is only available in the Russia Federation and some Commonwealth of Independent States (CIS) countries, like Kazakhstan.

Beside the tick population control, infections with TBEV are also tried to be prevented by the vaccination of vulnerable people. In Kazakhstan, forest workers and individuals that enjoy hiking in nature are eligible to receive a free vaccination by the Russian vaccination called “Encevir” (Microgen, Tomsk, Russia) that is based on the Far Eastern subtype related Sofjin strain. For the

expense of approximately €50.- also the Pfizer-produced “Tickovac” is available, that targets the European strain related to the Neudoerfl isolate.

Due to the danger of the need of destroying a massive stock of stockpiled Encevir TBEV vaccination in the early 2000s, the entire population of East Kazakhstan oblast underwent a mass vaccination campaign. East Kazakhstan was the oblast with the highest incidence values of infected patients in Kazakhstan at that time. This initiative had a stunning effect and since then the incidence for TBE decreased and stayed on a low level for many years. Only recently some TBE cases started to re-emerge due to the migration of non-vaccinated migrants from China to the area and the rise of an unvaccinated new generation in the last twenty years.

If all those prevention measures fail and a patient is infected with TBEV he may develop symptoms based on the strain of the virus and the inoculation dose of TBEV acquired during the infection process. The symptoms in patients can range from simple fever in its mild form to meningoencephalitis, polyradiculoneuritic or poliomyelitic symptoms in its strongest development (Gritsun et al., 2003).

In KAZ the fever form is predominant, similar to the European situation. However, some of the patients still develop the serious forms of the infection, depending of the physiological condition of the patient, the strain and as mentioned the inoculation doses of the received virus. Strains such as the Far Eastern and the Siberian subtype tend to initiate more severe courses. However, currently there is no standardised molecular biological method available in Kazakhstan to distinguish the strains of infected patients due to the lack of PCR analysis.

Nevertheless, if the fever in patients continues for many days or more severe symptoms arise, patients get admitted to hospitals. If patients consult a hospital with symptoms of fever or serous meningitis and it is in an endemic region and it is also the endemic period of TBEV that is from April to September, serum will be checked for the presence of anti TBEV reactive IgM and IgG antibodies. This check is performed with the Russian VectorBest TBEV ELISA kit. Only if the IgM screen turns out positive, the patient is categorised as a TBE case and the TBE clinical protocol of Kazakhstan gets initiated. This starts with a three to five-day treatment regime with anti TBEV human immunoglobulin. Based on the severity of the case, this treatment can be prolonged. This therapy is supported by supportive symptomatic treatment that aims to induce fever reduction. In most of the cases, patients recover after one to two weeks.

If a patient develops a more severe form of the TBE that also includes the involvement of the CNS is indicated by a spinal puncture. The CSF is then checked for traces of lymphocytes in the normally clear and cell free liquid. Lymphocytes automatically accumulate in the CSF since the meninges are directly connected to the spinal fluid system and hence all inflammation markers

that cause the symptoms of the meningism. If the cell titre in the CSF is higher than 25 lympho - cytes per µl of CSF isolate the infection is categorised as acute. If the numbers exceed the 100 cells/µl level, it is considered a more aggressive form of the infection. Serous meningitis patients are recommended to stay in hospital treatment until their CSF is again completely normal. To reach this level patients are usually checked in five to seven days, depending on patients' status.

In general, the treatment of TBE patients is similar all over the globe. Also, in *e.g.* European countries the main treatment regimen is symptomatic and supported by nonspecific antiviral and symptomatic treatment as interferons and steroids. However, the anti-TBEV immunoglobulin treatment is only recommended in Russia and some CIS states. Unfortunately, there are no meta-analysis that compare the impact of the Immunoglobulin treatment on the median recovery time of infected patients in comparison to the Western treatment.

Further it is difficult to draw a statistically sound claim, if the case fatality rates in TBE suffering patients is altered between European treatment options and the Kazakhstan treatment that also includes immunoglobulin. Worldwide TBE has a case fatality rate of 1-5%. In Almaty there were 40 reported TBE cases admitted to the hospital in 2011-2015. Out of those two succumbed to their infection, a 17-year-old young woman and a 40-year-old man. This would implicate a case fatality rate of 5% in the years from 2011 to 2015. The 17-year-old unvaccinated patient got bitten by a tick in the city of Almaty, immediately removed the tick and got a shot of emergency immunoglobulin treatment. Nevertheless, she developed three days later fever and seizures and got hospitalised. First, she was treated at the neurological unit, but since her symptoms worsened, she was transferred to the intensive care unit a day later where she died 27 days later. A pathological analysis revealed that she had severe brain edema. This was caused by the excessive reaction of the immune system of her body as a reaction to the virus infection. Only because the intensive care treatment she was alive for so long.

The male patient was only admitted to the hospital once he already fell into a coma after symp - toms of an infection for four to six days. He developed these symptoms approximately a two week after he was in the mountains for recreational reasons. However, he observed no tick bite during this event. In hospital the treating doctors were able to recover him from the cover, but anyways he died some 60 days later.

All the other 38 TBE patients in the mentioned before five years treated in Almaty city hospitals eventually recovered from their symptoms but unfortunately there are no long term follow up data available. Nevertheless, generally one can state that treatment of TBE cases, once detected, is comparable to international standard. However, the biggest issue remains the correct and efficient detection of infections in putative patients. Especially the complete absence of contemporary molecular biological methods such as a PCR analysis implies many weaknesses of the system.

This was also the initial motivation to start this broad investigation on tick-borne encephalitis virus in patients with serous meningitis and ticks in the Republic of Kazakhstan. For ticks, there is no information on the numbers of infected ticks in the distinct areas. PCR analysis or antigen screen is not routinely performed. For suspected patients there is only a simple IgM ELISA screen and no other diagnostic tools to determine the source of the disease. Beside this ELISA analysis there is no further diagnostic analysis in the patient, not to mention any molecular biological analysis.

This implies that there is a total lack on information about molecular biological details, there is no possibility to do a phylogenetic analysis based *e.g.*, on an E-gene sequencing. Further it is not possible to draw maps of endemic areas that show, where ticks have a high burden of virus and other not so dangerous areas. In Kazakhstan only human TBE cases are registered and this only allows indirect designation of highly endemic areas.

Beside a more convenient information on exact endemic areas one would also learn on the effectiveness of the employed vaccination by learning about the strains of the circulating TBEV. The Russian virus vaccine is efficiently working against the Siberian subtype but not that effective about the Far Eastern subtype. Still, the vaccine was administered for many decades without actually knowing if the vaccine is even qualified to protect from the circulating strains in the area. Along with an identification of the virus strain by sequencing goes also the understanding of the virulence of the virus and the potential implications for infected patients. Instead, ticks are annually intended to be eradicated by a massive chemical attack in vast areas of the country. Beside the ethical implications of such a chemical deployment, one could also reduce the area of treated regions by only spraying chemicals in tightly focused spots that have highly virulent strains.

Beside this lack of information of TBEV in ticks there is also an unsatisfactory high number of serous meningitis of unknown origin in treated patients in hospitals in Kazakhstan. It is suspected that many of the serous meningitis patients treated in hospitals are actually suffering from TBE disease.

Hence, we initiated a collection of first and second serum samples from hospital admitted patients with serous meningitis and combined it with CSF isolates. With these samples we were able to conduct a contemporary analysis of traces of TBEV in patients with many different methods. Each of the serological methods like ELISA or IFA have their downsides, but combined they improve the quality of the diagnosis. Strikingly however, in the 166 analysed patients we only identified five acute TBEV infections and two other flavivirus infected patients. Our initial hypothesis was that among the patients of serous meningitis with unclear aetiology the share of TBE would be much higher, and set at minimum 25% of the cases. Here we only identified 4% of the screened patients to have indications for a TBEV infection as possible cause of their symptoms. The reason

for the low number of found TBE cases in serous meningitis of unknown origin may rest in asymptomatic and fever forms of TBE.

In previous studies on patients with fever of unknown origin the share of TBEV was proven to reach up to 40 - 50% (Abdiyeva et al., 2020). However, in our study we only focused on serous meningitis patients with unknown origin. When only such patients are included, all mild TBEV infections that only cause fever are excluded. Hence you lose frequent strains such as the European TBEV subtype and you select for the Far Eastern and the Siberian subtype, as the latter result in a higher proportion of central nervous system involvement. However, still it was expected that the percentage of TBEV in meningitis cases in Kazakhstan should be comparable with investigations from other TBE endemic countries that are 20-30% (Mickienė et al., 2002, Laursen and Knudsen, 2003, Ergünay et al., 2011). Future research needs to deeper investigate into this finding.

For many patients there were also initial hospital diagnostic results available. Some of the hospitals were even able to identify a TBEV infection in patients on their own. Strikingly, however, we did not confirm the hospital diagnosis in two cases. This highlights the weakness of only employing an ELISA on IgM alone, with no further confirmation by other means. TBEV like all the other flaviviruses are notoriously prone to cross reactions and false positive results. Indeed, with one of the false positives patients there were no traces of flaviviruses at all and the diagnosis completely rested on a false positive result of the employed ELISA. In the second patient an actual infection with West Nile Fever was identified. West Nile Fever was never reported in the region of Almaty city before and hence was never on the attention list of practitioners. This initial finding now calls for a deeper investigation of the spread of WNFV in the region of Almaty to confirm that it is actually circulating there and these patients did not convey an imported infection. If it really turns out to be endemic it would be recommended to start screening patients with serous meningitis in the endemic period and endemic area routinely not only for TBEV but in parallel also for WNFV. The actual treatment for those two viruses is the same for the symptomatic treatment that is interferon and other antivirals. But the Kazakhstan specific immunoglobulin treatment is not effective in a WNFV and can be omitted in such an infection, since the IG treatment may cause severe allergic reactions in some patients.

For all the enrolled patients there was also a questionnaire available. This questionnaire was initiated to identify potential risk factors that drive TBEV infections. Since only ten flavivirus infections were detected in all the enrolled participants which is very small for a meaningful comparison of positive and negative cases. However, in the total cohort there were 31 patients that had an acute or previous infection or were infected with other viruses. By comparing those individuals to the remaining cohort, it became apparent that recurring tick bites raise the probability of getting a

viral infection. However, this finding is not surprising, since of course every single tick bite increases the chance of getting in contact with an infected tick. Such regression analysis of data from infected patients was also already performed previously in studies with similar scopes (Abdiyeva et al., 2019, Kiffner et al., 2010). They also found, that beside the number of experience tick bites also the living conditions play a role. The composition of the living environment such as occasional bushes and agricultural fields increased the risk of a TBEV infection. However, in our study, the living conditions were not significant. Furthermore, when looking at the sources of the data one learns that in the previous studies (Abdiyeva et al., 2019) mostly patients from rural areas were investigated while our human serology study focussed on patients from Almaty city and patients living in rural areas of East Kazakhstan and Akmola region. In the statistical analysis we did not do a further sub clustering of patients by residence in rural or urban areas because the numbers would have been too low to draw sound conclusions.

A very interesting finding was also that several patients had antibodies from a previous TBEV infection. This previous infection did not have any influence on the current formation of a meningitis in these patients. However, it gives a first idea on the rate of TBE cases in a population, without claiming the soundness of a full-scale seroprevalence study. In this study we found that three from 166 patients had IgG antibodies against TBEV or other flaviviruses and hence suffered from an infection without realising it. Another paper (Abdiyeva et al., 2019), performing similar screenings on patients with FUO found a previous infection rate of 1.24% in 802 patients. Combined one can draw the conclusion that in Kazakhstan the rate of persons that had a TBE in their lifetime is about 1.34%. This would be a very high number for a non-endemic region, but can be classified as low in an endemic area.

In all the studies performed in Kazakhstan, the predominant tick species transmitting the virus are from the genus *Ixodes spp*. or *Dermacentor spp*., a similar species composition as reported in other areas of Europe and Asia. Ticks are also notorious transmitters of lyme borreliosis. Indeed, in two out of the cohort of 166 patients a bacterial infection with Borrelia was diagnosed and treated with antibiotics.

To learn more about the actual infection rates of ticks in the nature and grasslands of Kazakhstan mass collections of the eight-legged blood suckers need to be conducted. As mentioned above, the numbers of ticks are annually counted by the local authorities and if numbers are too high, chemical countermeasures are initiated to reduce the numbers of ticks in recreational areas like parks and forests.

However, a contemporary analysis using molecular biological methods was never performed on ticks in Kazakhstan so far. So, there is no understanding of the percentage of TBEV carrying ticks and not talking at all of the subtypes of the virus. Including this study there are now two studies

investigating the virus load in ticks in different regions of Kazakhstan. In general, an oscillation of the tick numbers can be observed in 3-5-year intervals. The study conducted in 2016-17 was in a timespan, where relatively few ticks were active. Still in 35 sites they captured 2,300 ticks, pooled them into 500 units and identified there no TBEV positive fractions in the area of Kyzylorda (Abdiyeva et al., 2020). In a second attempt in Almaty region 1,700 ticks got captured and 22 turned out to be positive for TBEV in this approach. In the study presented here ticks were collected in 2018-19 and this was a phase of high tick activity. At 58 sites more than 10,000 ticks were collected and thirteen of those turned out to be positive for TBEV. Although there were more ticks analysed in the second study the number of positive ticks was higher in the screen from Almaty region. This is probably due to the environmental circumstances. The landscape of Almaty region is characterised by small mountains and high grass areas, perfect living conditions for ticks. Furthermore, in Almaty region are no chemical countermeasures against the ticks in force. Hence there are simply more ticks and also the number of infected ticks can increase. In our study, we collected ticks in Almaty city, an area that is actively eradicated of ticks by chemical efforts. The other areas are the region of Talgar, where two positive ticks got identified, Akmola where also two positive ticks were found and East Kazakhstan oblast, where nine of all the captured ticks turned out to be carrying a flavivirus. Regions of Talgar and Akmola have no chemical countermeasures in place. East Kazakhstan is known to be highly endemic for TBEV since many years.

With these positive ticks at hand, it is now finally also possible to start molecular biological analysis. All the positive ticks were submitted to molecular biological sequencing of the RNA information of the viruses to learn more about the strain and subtype of the species. By sequencing a short fraction of the E-gene we identified all the revealed viruses in the ticks belonging to the Siberian strain of TBEV. This strain can be further subdivided into subtypes. There a strong geographical affiliation could be revealed. All viruses isolated in Akmola region belonged to the western Siberian lineage. The Baltic lineage was identified in Almaty city, similar to the sequences identified in Almaty region by a previous screening study (Abdiyeva et al., 2020). This is a confirmation of a previous study in this region and highlights the quality of the applied methods. In East Kazakhstan region the Chinese lineage was dominating. This is not surprising, since East Kazakhstan is directly bordering to China and tick migration may be linked to bird migration in this cross-border regions. The same is true for the finding of the Western Siberian lineage in Akmola, the region around the capital of Nur-Sultan that forms the prolongation of the Western Siberian landscape. The Western Siberian lineage of TBE causes relatively mild symptoms in infected humans. Indeed, there are hospital reports about TBE diseases in Nur-Sultan and all of them

survived and had mild symptoms, confirming our finding of the predominant Siberian strain. However, some Russian papers report (Pinegina et al., 2013) also about severe cases in humans, but molecular information on the substrains are missing in those reports.

Contrary, for the identified Baltic lineage, a strain reported to cause mild to moderate disease outbreaks in humans, there are reports of fatal outcomes in Kazakhstan. As reported above, in Almaty city there were two patients that died from their TBEV infection in the last years. Since they got their infection in Almaty it can be assumed that their infection was caused by the Baltic lineage. However, unfortunately, from those two patients there are no sequences of the virus available to compare the sequences. There is only one report of a sequence derived from a patient in the Almaty region. It was a patient from the Alma-Arasan mountains region. The sequence was generated by Russian scientists and submitted to GenBank (KJ744033) (L'Vov D et al., 2014). The sequence also belongs to the Siberian strain, but in the paper there is no information provided about the subtype of the strain. This finding goes along with other unpublished initiatives that all identified the Siberian subtype.

Unfortunately, we were not able to initiate a full genome assembly of the identified strains. Unfortunately, we were not able to initiate a full genome assembly of the identified strains. The generated fragments were of too low quality to sequence and hence the sequencing failed. It was initially intended to get a full sequence of all the identified TBEV samples by a method dubbed primer walking. The full sequence of a virus would add additional information about the virulence of the viruses circulating in the regions. Some sequences in the UTR regions of the genes are known to further enhance the infectibility of a virus in human cells (Sakai et al., 2014). Furthermore, a comparison to other viruses in neighbouring areas would further improve the ability to draw migration maps of the virus throughout the regions.

Another major effort of the latter days of the project was the intention to grow the virus isolates in a BSL3 laboratory. Unfortunately, also all these efforts failed due to a bad quality of the cell culture cell lines employed. We intended to grow the virus on either VeroB4 or A549 cells. Since the cooling chain of the cells could not be maintained by the shipper at all times during the transport to the laboratory in Almaty, cell growth rates were insufficient and no virus could be produced in those lines.

Still, from the generated sequence information one can deduct several important insights on the situation of TBEV in the Republic of Kazakhstan. Now one can scientifically confirm that the employed vaccines that are administered in Kazakhstan are aiming at the correct virus strain. As long as the vaccination regimen is administered in the correct way, the vaccination can induce a high protection in individuals (Rampa et al., 2020). The vaccination regimen should follow the international recommendations that demand an initial shot in autumn or latest winter and then a

second following two to four weeks later. Repeat this vaccination a year later and then refresh the vaccination titre all three years. This vaccination regime is the same with the Pfizer or the Russian vaccine.

Furthermore, due to our study in patients with serous meningitis of unknown origin we did not find TBEV infection in patients in areas that are not officially endemic for TBEV, so there is no need to open the question about vaccinating the entire population of Kazakhstan in these regions. However, in the regions, that are endemic, inhabitants should actively be encouraged to get the vaccination. Although the case fatality rates are relatively low, an infection still has a high burden on the body and on the health system of the country. Hence, the vaccination status of all the residents in affected areas needs to be monitored and assumptions on the efficacy of the administered type of vaccination can be made by following up long term the wellbeing of the individuals.

Beside the vaccination there some other issues to be considered in the future in the public health system of Kazakhstan. There is the need to initiate a massive effort to screen all suspected patients fulfilling case definition criteria using PCR methods, especially in endemic regions. Further, since the counting of ticks is anyways conducted annually it would be easy to screen all tick pools for TBEV, not only in endemic regions but also in non-endemic regions. This would make the predictability of future hotspots of potential TBE cases more accurate and chemical countermeasures can be geographically better targeted and reduced to the absolute spatial minimum, which is favourable also from an ecological point of view. Furthermore, if in non-endemic regions TBEV positive ticks are found, effective diagnostics should be provided at the hospitals to find TBE cases more reliably and treat them correctly. This is a more effective way to improve the hospital treatment of prospective patients. Alternatively, there is only the option to offer TBEV screening in FUO patients all over Kazakhstan, however, this will be much more expensive than an efficient PCR screening in ticks all over the country.

Regarding the clinical treatment, the Kazakh recommendations are close to the international standards. Only the application of the anti TBEV human immunoglobulin should be thoroughly reconsidered by clinical studies if the benefit really outweighs the side effects. From literature review (Bröker and Kollaritsch, 2008, Kluger et al., 1995) it is apparent that also patients that do not receive the immunoglobulins have high chances to recover from the disease and the administration of Immunoglobulin might induce severe side effects. Some of the receiving patients develop severe allergies also leading to an allergic shock. However, on this case there are no meta-studies yet since too little official numbers of such side effects exist.

In summary based on our findings and the findings of others one can state that diagnostics based on ELISA alone are not sufficient. ELISA against antibodies detecting flaviviruses are prone for cross reactivity potentially leading to false positive results. Diagnostics of flaviviruses should

never alone rest on an ELISA but always be confirmed either by a combination of ELISA with IFA or with a contemporary and qualified PCR.

There are still high numbers of severe viral infections in Kazakh hospitals where the causing agent is not known. It is highly encouraged to initiate a broad screening of CSF isolates from all virus infected patients in hospitals. Information from CSF screening improves the quality of the differential diagnostics and supports the initial serological findings. So, for instance if the ELISA is positive and also the CSF samples are positive, the diagnostic can be confirmed. If the Serum based ELISA is positive but the CSF screening is negative, one has to be very cautious when making a diagnosis.

All these initiatives aiming at the treatment of patients in the hospitals, a broad bio surveillance initiative needs to be started on vectors with contemporary means. Ticks need to be screened for viruses and their subtypes need to be determined by molecular biological methods. This is especially important in areas where fatal cases in humans are reported. Future casualties can so be prevented and doctors in affected areas can be trained upon the subject on time. Doctors need to be encouraged to immediately get in contact with epidemiological laboratories once they have a suspected case of TBE, since so the virus can be isolated from patients and sequenced. Thereby it will be possible to compare the virus identity from ticks and patients and further learn about dangerous strains, the emergence of new hot spots and increase the health security level in the Republic of Kazakhstan.

5. Conclusion

Serous meningitis of unknown origin is a common diagnosis among hospitalised patients in the Republic of Kazakhstan. Serous meningitis can be induced by viral infections that are able to cross the blood brain barrier. A major driver of such diseases is believed to be Tick-borne encephalitis virus (TBEV), a member of the Flaviviridae family. However, there is a total lack of contemporary diagnostics to support this assumption.

Here we show that indeed TBEV in an agent for many cases of human meningitis in endemic and newly endemic regions in Kazakhstan. Beside that we found reactive antibodies against West Nile fever virus in sera from several patients, a virus that was previously unknown to circulate in Almaty oblast in Kazakhstan.

Performing molecular sequencing of the genetic information of the viruses highlighted that the TBEV in Kazakhstan belong to the Siberian subtype, and are clustering with the Baltic-, the Chinese- and the western Siberian subtype, according to their geographical location.

By analysing the genomic sequences of the virus, one finally can prove, that the vaccination applied in Kazakhstan is targeted and supports the protection from such an infection. However, we identified several wrong diagnosis results in the participating facilities, because standard laboratory diagnosis is based on a single ELISA result that is highly prone to cross reactivity. Here, hospitals should change their diagnostic panel and consider adding PCR analysis.

Further we encourage public health officials to intensively screen the local tick population in Kazakhstan for their prevalence in carriership of viruses. So, a good prognosis can be derived on infections that can be expected in humans.

In summary, our data confirm about the presumed activity of TBEV in Kazakhstan. Furthermore, they highlight the importance to initiate broad and nationwide longitudinal studies in human patients and in ticks on the prevalence of tick-borne encephalitis virus but also many other viruses, that can be transmitted by arthropods.

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Appendix

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

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ORIGINAL ARTICLE

WILEY

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

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 bi- phasic presentation. In the first viraemia phase the patient devel- ops 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 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).

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.

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).

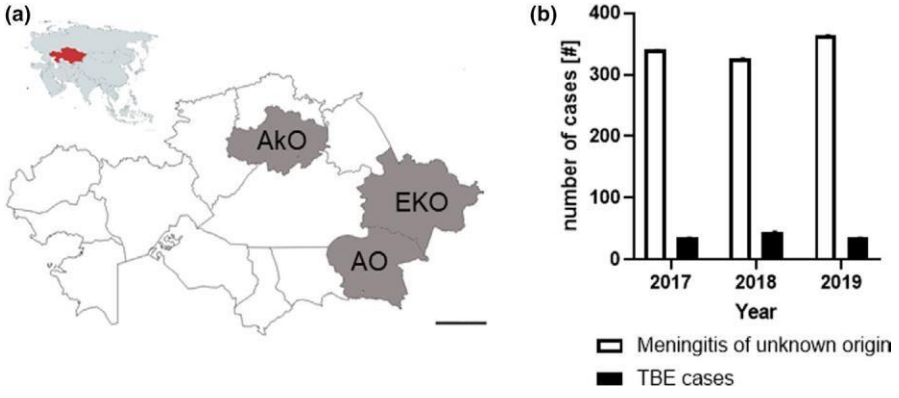

FIGURE 1 Overview on the situation of flavivirus infections in the Republic of Kazakhstan. (a) All patient samples were collected in three oblasts of the Republic of Kazakhstan, a country located in Central Asia (small map). The three oblasts were Akmola Oblast (AkO), Eastern Kazakhstan (EKO) and Almaty 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)

	Collected samples		
Region/hospital	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 ± 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

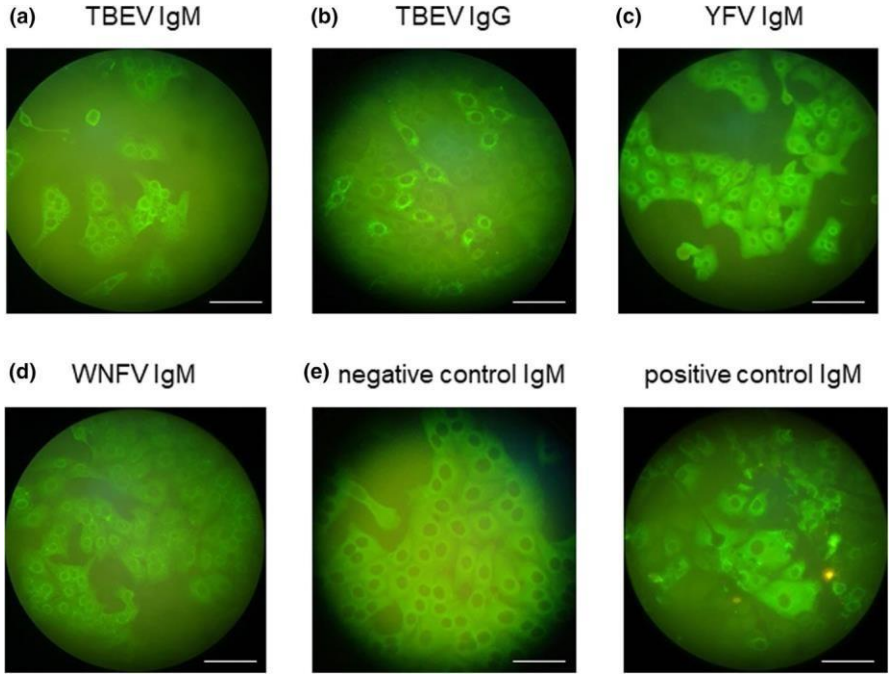

FIGURE 2 Representative images of the indirect immune fluorescence assay (IIFA). All ELISA positive sera were tested on an IIFA biochip to reveal any potential cross reactions by closely related other viral infections. Positive staining is shown for (a) TBEV IgM, (b) TBEV IgG, (c) YFV IgM and (d) WNFV 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 (Roelanddt 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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Statement on Pre-release and Contribution

The paper “Tick-borne encephalitis virus and West Nile fever virus as causes of serous meningitis of unknown origin in Kazakhstan. Zoonoses and Public Health, https://doi.org/10.1111/zph.12941” was published in March 2022 (Shin et al., 2022). As the first author I contributed to the conceptualization, the study design, data collection, collecting patients’ sera and CSF samples, coordinating further sampling with regional hospitals and doctors, capturing of ticks, identification and sorting of ticks, tissue homogenization and RNA extraction of samples under BSL-3 condition. I was responsible for the conduction of the serological investigation (ELISA, IFA, and Immunoblot) of samples and of the molecular-biological investigation (real-time RT-PCR, RT-PCR). Furthermore, I took care of the preparation and shipment of samples for sequencing, the coordination with the sequencing facility and subsequent sequencing data analysis and full data analysis. I took responsibility of writing the original draft, responding to the reviewers’ comments and reviewing and editing final version of the manuscript. All those steps were under the supervision of all supervisors and in coordination with the project team.

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List of publications

1. Tukhanova, N., **Shin, A.,** Turebekov, N., Nurmakhanov, T., Abdiyeva, K., Shev-tsov, A., Berdibekov, A., Sutyagin V., Maikanov, N., Zakharov, A., Lendyzish, I., Yeraliyeva L., Froeschl, G., Hoelscher M., Frey, S., Wagner, E., Peintner, L., & Essbauer S. (2022). Molecular epidemiology of Tula virus in Kazakhstan. Viruses.

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