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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 biphasic presentation. In the first viraemia phase the patient develops nonspecific flu-like symptoms. Only in this phase virus RNA can be detected (Veje et al., 2018). The second phase is characterized by involvement of the CNS with development of potential severe meningitis and meningoencephalitis (Lindquist & Vapalahti, 2008). During this phase the immune system creates first specific IgM and later IgG antibodies against TBEV. The severity of TBE varies from mild to severe with fatal outcomes depending amongst other 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).

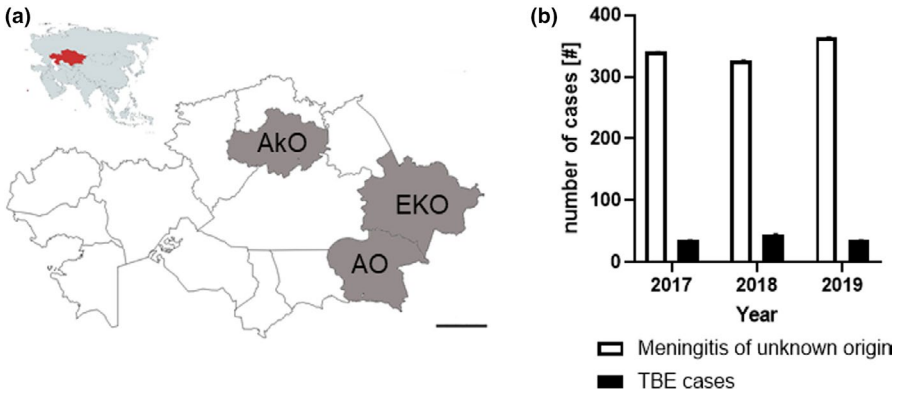

FIGURE 1 Overview on the situation of flavivirus infections in the Republic of Kazakhstan. (a) All patient samples were collected in three oblasts of the Republic of Kazakhstan, a country located in Central Asia (small map). The three oblasts were Akmola Oblast (AkO), Eastern Kazakhstan (EKO) and Almaty 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 ($SD \pm 11$) years. On 50 patients the hospitals performed routine laboratory tests, including bacteriological methods conducted in different types of samples, as well as agent specific PCR assays. In detail, for 28 patients (15.6%) an acute meningitis caused by *Enterovirus* was confirmed, for ten patients a meningitis caused by *Neisseria meningitidis* was diagnosed (5.6%), four patients had Human immunodeficiency virus in their serum (2.2%), one patient suffered from *Streptococcus pneumoniae* (0.6%), one from *Staphylococcus sp.*, one was positive for a mumps virus, one patient carried *Mycobacterium tuberculosis*, and one patient contained larval cysts of the parasite *Taenia solium*. Regarding to the TBEV endemic season 65% of patients were screened for TBEV immediately after hospitalization using a Vector Best IgM/IgG ELISA. For six samples an infection with TBEV was diagnosed by the hospitals as the IgM ELISA was positive.

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

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

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

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

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

(a) ELISA results

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

(b) Confirmation of ELISA positive results with IIFT

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

Abbreviations: JEV, Japanese encephalitis virus; TBEV tick-borne encephalitis virus; WNFV, West-Nile fever virus.

[a]One patient received TBEV-specific IgG for treatment and emergency prophylaxis.

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

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

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

3.2 | Serological analysis of sera and CSF samples

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

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

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

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

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

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

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

3.3 | Molecular biological analysis of CSF samples

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

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

4 | DISCUSSION

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

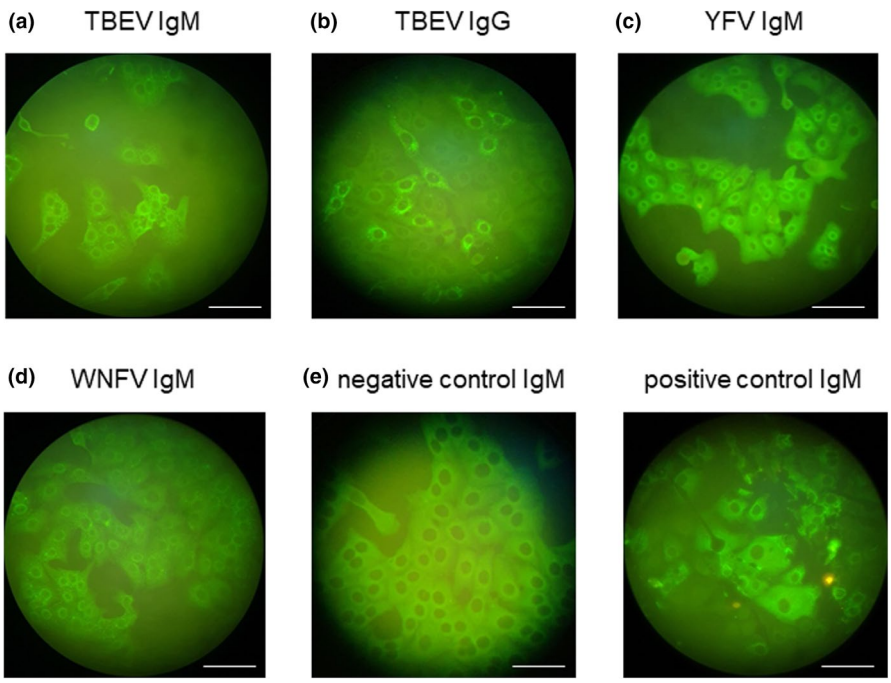

FIGURE 2 Representative images of the indirect immune fluorescence assay (IIFA). All ELISA positive sera were tested on an IIFA biochip to reveal any potential cross reactions by closely related other viral infections. Positive staining is shown for (a) TBEV IgM, (b) TBEV IgG, (c) YFV IgM and (d) WNFV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

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

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

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

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

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

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

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

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

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

However, the IIFA assay reacted positive in both sera on IgM. It is worth noting, that during a TBEV infection, IgM antibodies only appear approximately five days after the debut of the infection in blood and CSF and detection of TBEV RNA in CSF is at the same time rarely successful (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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