Out of the

Division of infectious Diseases and Tropical Medicine University Hospital, Ludwig-Maximilians-University (LMU) Munich

The investigation of highly pathogenic viruses in Kazakhstan

Doctoral Thesis

for the awarding of a Doctor of Philosophy (Ph.D.)

at the Medical Faculty of

Ludwig-Maximilians-Universität, Munich

submitted by

Karlygash Abdiyeva

born in

Almaty, Kazakhstan

submitted on

April 25, 2019

Supervisors LMU:	Title, first name, last name
Habilitated Supervisor	Prof. Michael Hoelscher
Direct Supervisor	Dr. Sandra Essbauer
4th LMU Supervisor	Dr. Norbert Heinrich

Supervisor External:

Local Supervisor Dr. Gerhard Dobler

Reviewing Experts:

1st Reviewer	Prof. Michael Hoelscher
2nd Reviewer	Dr. Sandra Essbauer

Dean: Prof. Dr. med. dent. Reinchard Hickel

Date of Oral Defense: November 12, 2019

Abstract

Background	Kazakhstan consists of favorable conditions for different vector transmitted infectious diseases such as Tick-borne encephalitis (TBE) and Crimean Congo hemorrhagic fever (CCHF). Up to 50 cases of TBE and 10 cases of CCHF are registered annually. Due to the lack of diagnostics approaches in remote areas many cases remain undiagnosed and the correct case numbers could be higher. With this study project I claimed to understand the infection rate of TBEV and CCHFV in ticks, to identify the circulating genotypes and find out the role of these infections among patients who suffer from fever of unknown origin (FUO).
Methods	In six districts of the southern part of Kazakhstan 2341 ticks were collected. RNA was extracted from the homogenates and all samples were screened for the presence of TBEV and CCHFV by real-time reverse transcription (RT-) PCR. Positive samples were amplified in conventional RT-PCR using pathogen-specific primers. Products of conventional PCR were sequenced using Sanger sequencing. In two regions the serological and molecular investigation of human sera from two pilot regions was conducted.
Results	TBEV RNA was detected in 48 out of 493 investigated tick pools. No positive tick pools with CCHFV RNA were detected. The picture of serological investigation of human sera among patients with fever of unknown origin shows that the antibodies to TBEV and CCHFV were detected in serum samples of 18 (2.24%) and 102 (12.7%) out 802 tested patients respectively. Molecular investigation revealed that CCHFV subtypes Asia1 and reassortant Asia1 and Asia2 and Siberian subtype of TBEV circulate.
Conclusion	For the first time comprehensive data on the prevalence of TBEV and CCHF in vectors and among patients with FUO based on molecular methods of investigation, the circulating of Siberian genotype of TBEV and reassortant of Asia 1 and Asia 2 of CCHFV in Kazakhstan were described. The new data will help to improve the surveillance system of these infections in Kazakhstan.

Key words

Tick-borne encephalitis virus, Crimean-Congo hemorrhagic fever virus, Siberian Subtype, tick, fever of unknown origin.

List of abbreviation

PCR	Polymerase chain reaction
CCHFV	Crimean-Congo hemorrhagic Fever virus
TBEV	Tick-borne encephalitis virus
ELISA	Enzyme-linked immunosorbent assay
IFA	Immunofluorescence assay
FUO	Fever of unknown origin
WHO	World Health Organization
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
Env	Envelope
S-segment	Small segment
L-segment	Large segment

Declaration of Published Contents

Parts of this thesis have been published in the peer-reviewed paper **Abdiyeva K**, Turebekov N, Dmitrovsky A, Tukhanova N, Shin A, Yeraliyeva L,Heinrich N, Hoelscher M, Yegemberdiyeva R, Shapiyeva Z, Kachiyeva Z,Zhalmagambetova A, Montag J, Dobler G, Zinner J, Wagner E, Frey S, Essbauer S. Seroepidemiological and molecular investigations of infections with Crimean-Congo haemorrhagic fever virus in Kazakhstan. Int J Infect Dis. 2019 Jan;78:121-127.

Content

List of tables

List of figures

1 CHAPTER

1.1 Introduction

More than 200 conditions could cause fever such as oncology, rheumatology, and infections. Of all the conditions the infections remain the main cause. Some causative agents as Rickettsia could be a reason of a long term fever, for some viral agents there fever duration is short (1). Despite the permanent development of diagnostics facilities, the causative agents of fever are not identified completely. The identification of the causative agents of fever are very important as fever could be a symptom of a serious infection and a sign of an emerging disease. Moreover, a long-term fever has an impact on health care cost. Examples of emerging diseases are arboviral diseases as Crimean-Congo Hemorrhagic Fever (2) and Tick-Borne encephalitis(3). Also, over the last years arboviruses were considered to have a significant burden on diseases among humans (4) and arboviruses were also considered to be a cause of fever in a certain proportion of patients (5). In the recent years arboviruses were also getting attention because an invertebrate arthropod vector is involved in their transmission (6) .

Within the recent years not only clinical aspects provoke the investigation of arboviruses in the territories of Central Asia. The analysis of global emerging pathogens showed that the territories of Central Asia have a moderate to high risk for pathogen emergence (6). Kazakhstan is one of the biggest countries in Central Asia and it borders with Russia, China, Uzbekistan, Turkmenistan and Kyrgyzstan. The population of Kazakhstan in 2018 was approximately 18.3 million people (www.forum.kz). Due to rapid economic growth, strengthened international business relations, expansion of tourism and globalization there is a high demand of knowledge on arboviruses circulation, their epidemiology, possible impact on health care and their genotypes (7).

Kazakhstan has intercontinental climate with a big range of temperature zones. The hottest month in Kazakhstan is July, the highest temperature was registered in July +49 ^{0}C in Turkestan oblast (former The South Kazakhstan oblast) and the lowest temperature in Akmola oblast -57 ^{0} C in the Central Kazakhstan. The landscape of Kazakhstan is varied, desert, semi-deserts, steppes, forests. Hence, the territory of Kazakhstan has very favorable conditions for different vectors of zoonotic diseases. Northern, Easternбand South-East parts of Kazakhstan are covered with forests area goes cross the mountains with enough humidity. The southern parts and western parts of Kazakhstan are mostly dry and the only source of water are big rivers. Such different landscapes are perfectly matching the preferable conditions of distribution of highly

pathogenic agents such as bacteria and viruses (8). Following this, according to reports of Kazakh Scientific Center of Zoonotic and Quarantine Diseases (KSCQZD) 40 %-80% of territory of Kazakhstan are vulnerable to highly pathogenic bacterial pathogens as *Yersinia pestis, Francicella tularensis, Bacillus antracis*. However, there exist almost no data on the prevalence of arboviruses in vectors and their proportion as a causative agent among the patients who suffer from fever. But three oblasts (areas) were designated as endemic for Crimean-Congo Hemorrhagic Fever and two oblasts (areas) are endemic for Tick-borne encephalitis (Atlas) (Figure 1).

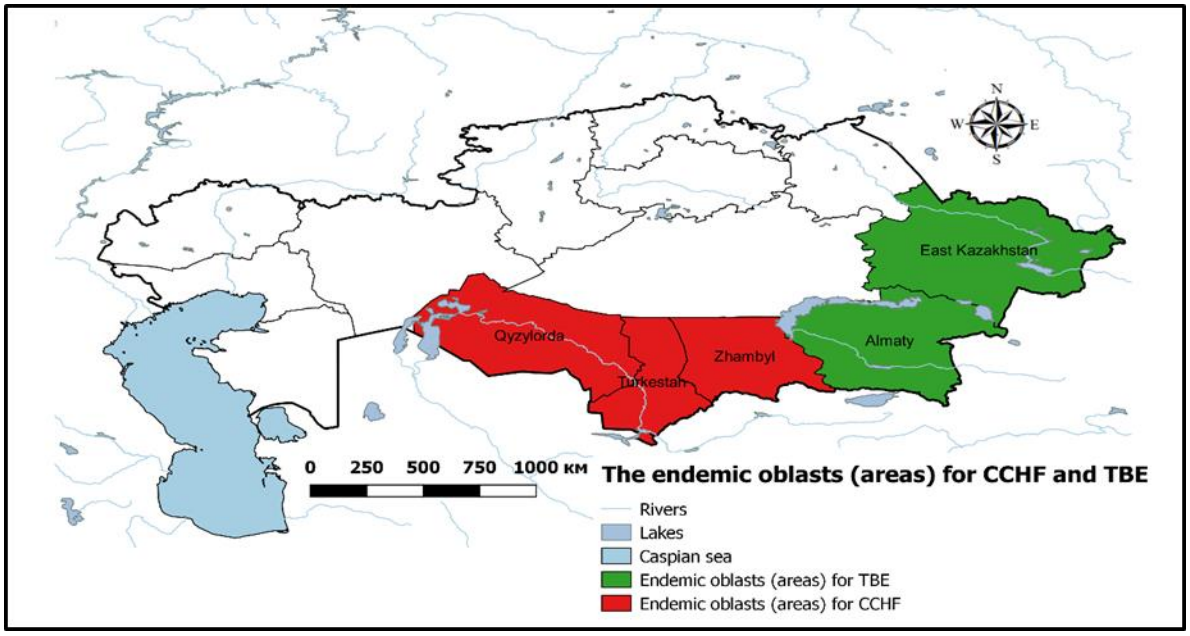


Figure 1. The endemic oblasts (areas) for CCHF and TBE in Kazakhstan

According to the endemic regions for CCHF and TBE two pilot oblasts were chosen for this study: Kyzylorda oblast in the south and Almaty oblast in the south East of Kazakhstan.

Kyzylorda oblast

Kyzylorda oblast is located east of the Aral Sea in the lower reaches of the Syr Darya River, mainly within the Turan lowland (height 50-200 m). The climate of Kyzylorda oblast is continental and extremely dry with long hot and dry summer and relatively warm, short snowy winter with little precipitation. The average July temperature in the north-west is 25.9 ° C, in the south-east it is 28.2 ° C, in January it is 9.8 ° C and 3.5 ° C, respectively. The amount of precipitation in the north-west off the coast of the Aral Sea is about 100 mm (the smallest amount of precipitation in Kazakhstan), in the south-east in the foothills of the Karatau up to 175 mm. Kyzylorda oblast consist of 7 districts

and two cities Kyzylorda and Baikonur. The population is approximately 753148 people (https://e-kyzylorda.gov.kz).

Almaty oblast

Almaty Oblast share the borders with Zhambyl Oblast to the west, Karaganda Oblast to the northwest (the water border runs along Lake Balkhash), and East Kazakhstan Oblast to the northeast. In the east, the region borders with China, in the south with the Republic of Kyrgyzstan (Chu and Issyk-Kul regions). Almaty oblast has a complex geographic characteristic and a very diverse relief. Almaty oblast is located between the ridges of the Northern Tien Shan in the south, Lake Balkhash in the northwest and the Ili River in the northeast; in the east Almaty oblast is bordered by China. The entire northern part of Almaty oblast is occupied by South Semerechiye plain weakly inclined to the north (height 300-500 m), crossed by dry channels - Bakanas, with arrays of ridge and loose sand (Sary-Ishikotrau, Taukum). The southern part is occupied by mountain ranges up to 5000 m high as Ketmen, Zailiysky Alatau, and Kungei-Alatau. The northern part of Almaty oblast is characterized by sharp continental climate, relatively cold winter up to –35 ° C, hot summer up to +42 ° C. Precipitations are only 110 mm per year. In the foothill zone the climate is mild the precipitation is up to 500-600 mm. The amount of rainfall reaches 700–1,000 mm per year. The length of vegetation period in the foothills and on the plains is approximately 205–225 days. The north and northwest are almost devoid of surface runoff. The only river here is Ili, forming a highly developed marshy delta and flowing into the western part of Lake Balkhash. In the south, the foothills of the river network are relatively dense; most of the rivers (Kurty, Kaskelenka, Talgar, Esik, Turgen, Chilik, Charyn, etc.) originate in the mountains and usually do not reach Ili River. In the mountains there are many small fresh lakes (Big Almaty and others) and mineral springs Alma-Arasan. Almaty oblast consist of 17 districts. The population of Almaty oblast is approximately 2017277 people (http://zhetysu.gov.kz/ru/).

Ticks and sera were collected from these two pilot oblasts (see Figure 5).

Despite the recognized endemic regions for CCHF and TBEV, the Kazakh surveillance system on these infections relies on passive population surveillance and registering the clinical cases reporting from the health care providers (Hospitals). Due to the primitive system of diagnostics based on serological and molecular methods of investigation, there is lack of data on the exact prevalence of CCHFV and TBEV in vectors and circulating genotype. Serological and molecular methods of investigation also need improvement and upgrade to the modern and worldwide used methods. The main diagnostic deficiency is not registering the real data on these infections. Also due

to the lack or in some regions the absence of diagnostic assays and lack of knowledge of medical staff the cases of TBE or CCHF could be remained unrecognized and unregistered. According to hospital data, there are many suspicious cases of TBE or non- hemorrhagic form of CCHF after tick bites.

Therefore, a good understanding of the prevalence of CCHFV and TBEV in vectors, epidemiology of these infections, possible role of CCHFV and TBEV in a proportion of patients who suffer from fever and updating the modern diagnostic assays are need to prevent the distribution of these infections in Kazakhstan and surrounded territories. Moreover, this knowledge will improve the surveillance and health care system of Kazakhstan. The aims of this study are to determine the prevalence of CCHFV and TBEV in patients with fever, to assess the demographics associated with fever, to know the prevalence of CCHFV and TBEV in vectors of pilot oblasts (areas) and to identify the circulating genotypes of these arboviruses.

The research project was conducted in two directions. First, tick study or investigation the prevalence of TBEV and CCHFV in ticks collected in pilot regions. The tick collection was conducted at 32 locations of two oblasts (Almaty and Kyzylorda oblasts) in May and June in 2015. The second direction of study was serological investigation of 805 patients with fever to detect the presence of antibodies against the CCHFV and TBEV. The blood was collected in pilot regions from April till October 2014-2015, during the tick activity. The serological study was also accompanied by epidemiological survey. The methods of investigation are outlined in detail bellow.

2 CHAPTER

2.1 Literature review

2.1.1 Literature review. Tick-borne encephalitis (TBE)

2.1.1.1 History of TBE

Since the early 1930s, in in the Far East of Russian Federation, medical personnel faced with severe acute illness with damage to the central nervous system, often ending in the death of patients. The disease was completely unexplored. In 1935, neuropathologist A.G.Panov, who worked for the Far East, for the first time found that this “mysterious” disease is encephalitis. The etiology of this encephalitis was not known. But he considered that this encephalitis is already known famous Japanese encephalitis. His attempts to investigate the possible etiological pathogen in “the Far Eastern Paster” station did not lead to success. It was not only the time of wide economic development of the Far East but also the time when the Soviet Union had an external threat from militaristic Japan. Manchuria was seized by militaristic Japan and developed a plan of military action “OTSU” against the USSR ". Due to this threat, there were placed large military units that stood right in the taiga. Among the soldiers, the number of patients with unknown neuroinfection was grown. Epidemiological trouble could negatively affect the defense capacity of the Far Eastern region(9). People's Commissar of Defense Marshal K.E. Voroshilov asked for help the health care ministry of the Soviet Union. Therefore, the preparation for an expedition to the Far Eastern region is started. The expedition was entrusted to lead the head of the country's first medical virologic Laboratory (1935) Professor Lev Zilber. L.A (10). The opportunity to select any specialists for the expedition, whom he considered is necessary was given to Zilber. Professor Zilber took exclusively the youth and did it quite consciously. He wrote later: “Of course, I gathered and warned of the dangers and difficulties or everything else but young people have in my eyes a huge advantage. They were not bound by old fallacies in regarding this disease ". They planned of investigation of this unknown encephalitis that was very successful. The first plan of research was in a case if unknown encephalitis is Japon encephalitis, the second plan of research was organized for a case if the unknown encephalitis is another encephalitis. And finally, the third plan of the research was for a case if the disease is not at all an encephalitis. These plans were developed in detail and brought success(11). During this expedition was detected the connection between the vector a tick *Ixodes persulcatus* and illness. During this expedition was detected the connection between the vector a tick *Ixodes persulcatus*

and illness. L. Zilber observed the connection, but Chumakov proved it during the experiment. This discovery was led into the prophylaxis measurements against the tick bites or tick-control measurements. Simultaneously, the young scientists Chumakov, Levkovisch, Shubladze, and Soloviev isolated the virus from the cerebrospinal fluid of patients suffering from the encephalitis during the acute fever, in average the virus was isolated from 29 patients. One of the most achievements of this expedition was not only virus identification, but also the description of the clinical presence by a young neurologist Shapoval, the description of pathologic changes during the autopsy of patients who died from this encephalitis by pathologist Kestner. During the autopsy, Kestner identified that the nervous system, especially the brain is susceptible to damage. During this expedition, for the first time, the treatment with the immunoglobulin was implemented by Shapoval and had a positive outcome in patients. Chumakov could get the Immunoglobulin from the convalescence blood serum(12).

During this expedition was for the first time described the laboratory infections by this virus. During the autopsy of the deceased patient, Chumakov M. injured his finger and got Tick-borne encephalitis, with severe clinical presence. At the same time, young scientists Soloviev V. and Gnevisheva E. were infected by tick-borne encephalitis and got a severe infection that affected all their life. Within three months, the expedition is started on 15 May in 1937 and finished on 15 August in 1937, the scientists of this expedition made a huge discovery in difficult conditions of taiga of Far-Eastern region. On 20 August of 1937, the results were reported by Zilber L. and the disease was named as spring- summer epidemic encephalitis or tick-borne encephalitis. L.Zilber was persecuted for political reasons and sent to prison till 1944. In 1938 the second expedition with Pavlovsky as a leader of this expedition was sent to the taiga of Far Eastern region. During this expedition, the circulation of the tick-borne encephalitis virus and its possible hosts and vectors were investigated, moreover during this expedition the first attempts of inactivated vaccine was established. However, some scientists were infected during work and died. In 1939 the third expedition, during which the first epidemiological experience on the vaccine against the tick-borne encephalitis was conducted by Levlovich E. and Dankovskiy N., also the new natural tick-borne encephalitis foci were discovered in Ural region and surroundings of the Ural region. This expedition also has its victim, during the laboratory work a young parasitologist Pomerntsev B., was infected by tick-borne encephalitis virus and died. The fourth expedition in 1940 with a scientific leader Levkovich E. was targeted for vaccination the

population under the group of risk. The immunization brought its positive outcome as decreasing and in some communities the liquidation of morbidity.

Despite the first strain isolation in 1937 by a group of scientist in the Soviet Union, the first reported case and detailed description of the disease consistent with Tick-Borne encephalitis virus was made by Schneider in 1931 in Austria(13). He described this disease as “meningitis serosa epidemica” of unknown origin and only in 1957 the etiology of unknown meningitis was described and identified that it was tick-borne virus. And at the beginning of 1973 the first experimental vaccine was ready(14).

2.1.1.2 Microbiology of TBE virus

He described this disease as “meningitis serosa epidemica” of unknown origin and only in 1957 the etiology of unknown meningitis was described and identified that it was tick-borne virus. And at the beginning of 1973 the first experimental vaccine was ready(15).The genetic material of the tick-borne encephalitis virus is 11kb single-stranded positive-sense RNA. The viral RNA consists of one long open reading frame, that encodes the mentioned above proteins (three structural PrM, C, E proteins and seven not structural NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), also 5’ type 1 cup and 3’ not coding regions (3’ NCR(16). The 5’ NCR is more conservative in comparison with 3’ NCR. 3 ‘non-coding region consists of a conserved region and variable region (V 3’ NCR) as well (17).

Based on E sequence and full genome three genetic lineages were distinguished, the European, Siberian and Far-Eastern subtypes. However recently this classification was reconsidered and now five genetic lineages are distinguished(18). So far, these subtypes are European, Siberian, Far-Eastern, Baikalian and Tibetian. Each of them has its own geographical distribution (19). Hence, Far Eastern subtype is mostly distributed in regions of the Far East of Russia, China and in some regions of the European part of Russia. In contrast, European subtype occurs in countries of Europe, in the European part of Russia and very rare in the Far East of Russia. Siberian subtype mostly is widespread in the territory of European and Asian part of Russia and very rare in European countries (19) (20). The new Baikalian subtype is distributed in the south of Eastern Syberia and North Mongolia (15). The Himalayan subtype is distributed in China in Qinghai-Tibetian Plateau (17).

Different subtypes of TBEV are related to different vectors (Ixodidae).

2.1.1.3 Clinical presence and treatment of TBE

The clinical presence of Tick-borne encephalitis is varied from the asymptomatic to the heavy clinical manifestation with the involvement and damage of the central nervous system. However, according to some authors, most cases of tick-borne encephalitis are asymptomatic. The proportion of patients with asymptomatic clinical presence ranges between 70-98%(20). The clinical presence of Tick-Borne encephalitis depends on a causative subtype. The course of Tick-borne encephalitis could be biphasic in a case the disease is caused by a Western type of virus and monophasic febrile illness in a case the disease is caused by Far-Eastern type of virus (21). However, this distinguishes needs more deep investigation due to the discovery of the new lineages of tick-borne encephalitis virus (18). But more authors observed that biphasic course of the disease occurs in 75% of all patients who suffer from the tick-borne encephalitis caused by European subtype of the tick-borne encephalitis virus. Moreover, it was observed that patients with a monophasic course of the diseases in the preponderance of tick-borne encephalitis cases have the involvement of the central nervous system as meningitis, encephalitis and meningoencephalitis as well. And only a not huge proportion of such patients have a “febrile headache,” but without the involvement of central nervous system, in literature, it is also known as an “abortive form” of the disease(22)(23). So, there is a distinguishing of tick-borne encephalitis according to the clinical manifestation as asymptomatic infection in 70- 98 % cases of tick-borne encephalitis and symptomatic infection as well. Symptomatic infection occurs in all age groups (22)

After the incubation period, that is lasting approximately between four and 28 days, but on average up to 10 days (20).The first stage of the disease is presented; it is also called as the initial phase of the disease. This stage is characterized by the common symptoms of intoxication and not specific symptoms as muscle pain, headache, fatigue, weakness. During this stage, the temperature is lasting on average up to 7 days. This stage of clinical manifestation is presented only with no specific symptoms and without the involvement of the central nervous system(23). The blood picture could have some changes. Due to the immune system of organism faces with viremia during the initial phase of tick-borne encephalitis, in the blood picture as leukopenia, thrombocytopenia alteration could be detected. (24). The initial phase could be abrupted on this phase but could develop into the second febrile phase. But the second febrile phase follows the initial stage after the afebrile, relatively asymptomatic period or some patients note the improvement in their condition(20) but some papers reported mostly in Russian

language (Kanter V., 1965; Leonova G., 1987) that tick-borne encephalitis caused by Far-Eastern Subtype doesn't have the interruption as a relatively afebrile period and have only monophasic course of the disease(21). The second febrile phase is characterized by central and peripheral nervous system involvement. The damage of the central nervous system is characterized by meningitis, meningoencephalitis, and meningoencephalomielitis and with involvement of the peripheral nervous system. It was recognized that tick-borne encephalitis could be presented with various clinical signs of the nervous system damaging and it was differentiated into the various clinical forms as meningeal, meningoencephalitic, febrile, poliomyelitic, polyradiculoneurotic and chronic as welll(25)(26).

Meningitis is the most frequent clinical form of tick-borne encephalitis (26). Meningitis is characterized by high fever, vomiting, vertigo, strong headache with photophobic suffering pain in the eyes, signs of meningeal irritation (26). Meningoencephalitis is also known as meningoencephalitic form of tick-borne encephalitis. Usually, it is combined into the meningoencephalitic form and polyomyelitic form into the meningoencephalomyelitic form(26). The meningoencephalomyelitic form of tick-borne encephalitis is presented by fever, not specific symptoms of common intoxication, signs of meningeal irritations and the signs of the brain substance damage that lead to the paralysis. (27). (27) (21) (28). The paralysis in meningoencephalomyelitis is often presented by flaccid poliomyelitis like paresis, and this type of paresis commonly affects the upper extremities as the shoulders, arms, muscles of the head elevators (27). In severe case the outcome of the disease form could be the death, the death could occur in 7 days after the onset of neurological signs and especially in a case of the bulbar involvement and diffuse brain edema. 30% of cases of this central nervous system sequelae are ended by death (90). (27) (25). In some proportion of patients occur as the abortive form of tick-borne encephalitis (91). This form is presented with clinics of febrile illness without the involvement of the central nervous system. The data over this form is limited, in Europe, only 2 % of patients develop this form (88), however, in Russia and Kazakhstan, this form is included in the commonly accepted classification and called "Febrile form of tick-borne encephalitis." It was classified differently into the separated form as the incidence of this form is up to 50% (Ustinova et al., 1997) (91) (77). The special form of tick-borne encephalitis is a chronic form. The chronic form is characterized by progressive physical deterioration and frequently accompanied by the alteration of mental health. The decline of physical health includes the damages of the nervous system. The clinical manifestation of chronic tick-borne encephalitis is varied

and could be manifested by Kozhevnikov's epilepsy, progressive neuritis of the shoulder plexus, lateral sclerosis, dispersed sclerosis, a Parkinson like a disease, progressive muscle atrophy(26). (Pogodina et. al 1986, Votyakov et al., 1976)(25)(26) . (29). (95).

Tick-borne encephalitis has a significant impact on public health (30) as the post-encephalitis syndrome is developing after the acute tick-borne encephalitis. This syndrome is characterized by flaccid paresis, damages of cranial nerves as hearing loss, tinnitus and disturbance of vision. (31). (31). (32). (26) In average 10% of patients develop the permanent paresis, although in Russian publications the developing of permanent paresis in 25 % of patients was reported.

Treatment of tick-borne encephalitis is complicated and depends on the severity of clinical manifestation. There does not exist the specific treatment of antiviral treatment. Patients require the detoxication therapy for water and electrolytic balance but with the strict diuresis control. Also, the supportive therapy requires administration of antipyretics, analgesics in a case of high temperature and pain. In a severe case with the cerebral nervous system involves the administration of anticonvulsive therapy is necessary. The critical patients due to the neuromuscular paralysis and involvement of the medulla oblongata have the respiratory failure. Therefore, these patients need intubation and assisted ventilation. One of the dangerous complications is brain edema. Brain edema is usually a bad prognostic sign and could be ended in death. The therapy against this complication typically prescribed a dehydration therapy and an administration of steroids (22) (22). Despite the absence of the specific treatment against tick-borne encephalitis recognized worldwide, in Russia and Kazakhstan the intramuscular Immunoglobulin is recommended in a case of tick-borne encephalitis (The recommendations of TBE treatment in Russia, 2013))(33). It is clear that the administration of Immunoglobulin against tick-borne encephalitis virus is dangerous as the Immunoglobulin could transmit HIV, the viruses of hepatitis B, C, D and could be a cause of severe allergic reactions. And the treatment with human immunoglobulin is no longer available in European countries but due to the circulation of Siberian strain and Far-eastern strain of tick-borne encephalitis that have a high tropism to nervous cells the intravenous and intramuscular human immunoglobulin against tick-borne encephalitis is recommended in high doses (Zaharcheva et al., 2013). In 2013 in Russia was conducted the study over the efficacy the Immunoglobulin. It was demonstrated that The Immunoglobulin efficacy correlates with the form of tick-borne encephalitis, the severity and the most important with the time of immunoglobulin administration. It was noted the rapid involution of neurological symptoms especially in patients with

encephalitis with focal symptoms. Also, the sanitation of cerebrospinal fluid was earlier; the post-encephalitic syndrome is shorter without severe impairment of the central nervous system was significant in comparison with the control group ($p<0.05$). (Zaharcheva et al., 2013). The new schemes of treatment with Immunoglobulin was suggested and later approved and accepted in the Russian Federation.(34).

The human intramuscular immunoglobulin is also available and recommended by the Kazakh Health Care system (*Recommendation for Tick-borne encephalitis treatment, The protocol #9 from 16 August 2016*) (34).

The experimental study on a mouse model (Knockout CD8) demonstrated that the immune system plays a substantial role in TBE developing. (35). This experiment highlighted the importance of the immune system as one of a crucial mechanism of cell-damaging and the new ways of the ideal treatment of tick-borne encephalitis. The ideal treatment should enhance the immune response against TBEV, but at the same time to suppress the damaging effect of the immune system. Also, one of such treatment is high doses of intravenous generic immunoglobulin against tick-borne encephalitis virus could be. (35). Despite the fact of limited validated data over the Immunoglobulin administration in some countries it is highly recommended. Hence, even though the studies demonstrate the efficacy of high doses of immunoglobulin the administration of human Immunoglobulin against tick-borne encephalitis is under the big debate and requires further validated studies on the efficacy and risks of it

2.1.1.4 Vaccines against TBEV

The vaccination is the most useful measurements again tick-borne encephalitis. The active immunization against tick-borne encephalitis virus decreased the incidence of tick-borne encephalitis in 1940 in the Far Eastern region of Russia and 70s in Austria(14)(26). Currently, four vaccines are available FSME-IMMUNE (Baxter/Pfizer, Austria), Encepur(Novartis, Germany), EnceVIR (Tomsk, Russia) and IPVE (Moscow, Russia) although the fifth vaccine was produced in China by Changchun Institute of Biological Products(36). Some countries with endemic regions for tick-borne encephalitis implemented the vaccination program regulated by Government. These countries are Russia, Austria, Finland, Hungary, Latvia, Slovenia, Switzerland, and Italy (30). In Kazakhstan only, the groups of risks in endemic regions are under the vaccination program (Recommendations on TBE, 2016, Health Care of Kazakhstan). Two vaccines FSME-IMMUNE (Baxter/Pfizer, Austria) and Encepur (Novartis, Germany) are based on European strains of TBEV, two vaccines EnceVir (Tomsk,

Russia) and IPVE (Moscow, Russia) are based on Far-Eastern strains of TBEV, and Chinese vaccine is also based on Far-Eastern strain of TBEV. In detail FSME-IMMUNE (Baxter/Pfizer, Austria) based on Neudorfl strain, this strain was isolated from the ticks *Ixodus (I) ricinus* collected in the surroundings of Vienna in 1971(37) Encepur (Novartis, Germany) based on Strain K23, this strain is also isolated from ticks i.ricinus in 1975. EnceVIR (Tomsk, Russia) is based on strain 205, this strain was isolated also from ticks, but from *I.persulcatus* in Khabarovsk region in 1975(36) while IPVE (Moscow, Russia) vaccine based on Sofjin strain isolated in 1937 in Far-Eastern region from the patient's brain (36). Chinese vaccine is also based on a strain with high homology with Sofjin strain and isolated from the patient's brain in 1953(38)(36). The data on the Chinese vaccine is limited. Moreover, this vaccine only was registered and administered in China.) (33). (36). Moreover, the experimental studies on the efficacy of these vaccines against the all known strains found out that vaccines FSME-IMMUNE (Baxter/Pfizer, Austria), Encepur (Novartis, Germany), EnceVIR (Tomsk, Russia) and IPVE (Moscow, Russia) induces neutralizing antibodies in protective titers against all known strains. (39). Despite the all vaccines induce the cross-protective immunity for all known strains, the phylogenic diversity could slightly influence efficacy and the strain peculiarities as well. Recent experimental studies demonstrated that E protein could be used as the target part for a recombinant vaccine against TBE developing. There was a developed an experimental vaccine including the recombinant TBEV E protein domain III immobilized on a dextran. It was noted that sequences of domain III are conservative among all flaviviruses and the neutralizing antibodies were found against this domain in sera. Due to these properties, the E Protein was a proposing part of tick-borne encephalitis virus for a vaccine and new diagnostics kit developing. However the protective efficacy was not proved, but it is a basis for further vaccine approaches developing(40).

2.1.1.5 Biosafety containment of TBEV

All diagnostics methods except the work with a virus cultivation could be provided in laboratory Biosafety level 2, but the clinical samples should be inactivated by AVL (Qiagen, Germany) for molecular methods of diagnostics or by heating during 560 C during 30 min for further serological investigation to according to the recommendations(41)(42)(43)(44)

2.1.1.6 Diagnostics of TBE

I described the methods as we developed SOPs on diagnostics during the project study and they were implemented in KSCQZD.

Rapid and timely conducted of TBE diagnostics is important. Currently, the diagnostics based on molecular methods and serological methods investigation are available.

According to recommendations of European center for disease control meeting report from 2011, TBE diagnosis demands both clinical symptoms and laboratory findings as the detection of viral RNA in clinical samples or IgM and IgG in serum, or IgM and IgG in cerebrospinal fluid (45). In a case of epidemiological surveillance of TBEV natural foci, ticks collected in fields or collected from the livestock, wild animals, rodents are investigated for the presence of TBEV RNA. In some cases, the organ homogenates of wild animals usually rodents are investigated for the presence of the viral RNA. In the endemic region for TBE, the blood of the livestock could be tested for the presence of antibodies against TBEV(46).

Molecular methods of investigation

Previously, the detection of viral TBEV RNA was detected in ticks and clinical specimens separately. For detection, the viral TBEV RNA in ticks the nested RT- PCR (120) (121) (122) and the RT-PCR based on TaqMan chemistry (123) were used. At the same time, the applying RT-PCRs assays in detection of viral RNA in clinical specimens were also developed and performed as the nested RT-PCR assays (124) (125).

Currently, protocols updated for different commercial chemistries and targeted to detect E gene of TBE viral RNA are available. (126).(127). The modern molecular methods of TBEV investigations must be targeted to the rapid and accurate detection of the viral RNA in a material as in clinical specimens and ticks for epidemiological observations as well. In 2013 was presented a novel RT-PCR assay based on TaqMan chemistry (47), the assay was developed to detect all known subtypes of TBEV as the maximum homology within 3'non- coding region of four strains Neudoerfl, Vasilchenko, strain 263, and strain Hypr (119). This RT-PCR was used in our research project

Serological methods of investigation.

The diagnostics of tick-borne encephalitis in clinics are mostly based on serological methods. The applying of this approach in clinics are explained by the developing of the disease and changing the phase of viremia (first 1-6 days) by the following phase of immune response and antibodies releasing (48). Patients usually are admitted to the hospital during the second phase of the disease, when the neurological symptoms

occur and to find the RNA of TBEV is rarely successful. Also, it is more likely that antibodies IgM against TBEV would be detected approximately on and after the sixth day after the onset in serum and after six weeks the activity of IgM is declined. IgG antibodies appeared in serum later and could be revealed up to one year. In cerebrospinal fluid, IgM antibodies could be detected from the 9th day after the disease onset(45). Therefore, in hospitals, the serological methods of investigation of TBEV are prevailed (48). Currently, various diagnostics tests form the serological methods of investigation. One of the widely used is the enzyme-linked immunosorbent assay (ELISA). Currently, there are many available commercial kits on detection of IgM and IgG antibodies (49)(50).

2.1.1.7 Epidemiology and vectors of TBE

Tick-borne encephalitis is a significant health care problem. Annually up to 15000 cases are registered annually (51). People are infected through the tick bites or consumption of raw infected milk. The domestic animals are the host for ticks *spp. Ixodes*. Hence, ticks infect animals. Despite the livestock do not show any clinical signs and do not develop the disease, infected animals play a substantial epidemiological role as they are a source for the so-called alimentary acquired TBE (52).

The main reservoir for the virus circulation in nature are small mammal species as they can transmit the virus through the viremia and can infect other ticks through the co-feeding and feeding. It was observed that certain rodents are an important reservoir for TBEV. Moreover, the reservoirs are distinguished by circulating virus subtype. For example, in Europe with European subtype, the rodents *Apodemus (A.) flavicollis, A. sylvaticus* , and *Myodes* spp. are the main virus reservoir (13) whereas in Siberia with Siberian subtype are *A. peninsula* and *A. agrarius* and in Far-Eastern region with Far-Eastern subtype *Myodes rufocanus* and *Microtus arvalis* respectively. After discovering the new two subtypes Baikalian and Tibetian (18), the main reservoir of these subtypes is still unclear. However, the number of mammalian species that could be a host for ticks are significant.

The tick parasites the host animal according to its stage development. Larvae and nymphs are preferring to parasite and feed on small forest animals, mainly on mouse rodents or on medium sized animals. However, the range of animal's size could be varied to large hosts. Imago parasites mostly on medium size and large hosts (53). Ticks could be infected not only through feeding and co-feeding but also through a transovarial transmission. In the territories of western Europe countries, Turkey and the

territories expanding to the southeast to Caucasus and Iran the main vector of TBEV is ticks *I. ricinus* , whereas in the territories of eastern Europe countries, Japon, China, Mongolia (54) and Kazakhstan the main vector of TBEV is tick *I. persulcatus*. *I. persulcatus* and *I. Ricinus* could co-circulate on territories of Russia, in some countries of Eastern Europe, Estonia and Latvia (27)(55). In Siberia and far-Eastern region, the third species similar by ecological features is *I. pavlovsky*. Currently ticks *I. pavlovsky* and hybrids I*. pavlovsky* with *I. persulcatus* are becoming the main vector in Siberian surroundings (56) However other tick species could be a vector of tick-borne encephalitis. Recently, it was observed that in some natural foci of TBEV in Russia the main vector of TBEV tick spp. *Dermacentor*. The circulation of the virus is supported by tick spp. *Dermacentor.* For example, the main vector of TBEV in the Republic Altai of Russia is tick spp. *Dermacentor*. Due to the absence of tick I.persulcatus in these territories the main vector of tick-borne encephalitis is *Dermacentor nuttalli*. However, this tick species is more prominent and therefore not so mobile as ticks *I.persulacatus,* and usually, people could remove them before a bite (Shuchinova et al., 2013) (46).

The epidemic potential of natural foci of TBEV is varied and depends on many factors as landscapes, peculiarities of circulating virus (mostly subtype), the density of vectors and their infection rate. One of the critical factors is the weather conditions and climate, as the weather condition influence the density of hosts. Moreover, it was observed that *I. ricinus* and *I. persulcatus* depends on the humidity level. Thus, *I. ricinus* and *I .persulcatus* successfully adapted to different types of forests in the temperate zone of Eurasia. These species have high ecological plasticity, which is manifested in their ability to successful existence in several types of forest formations from the extreme north of the taiga zone and deciduous forests in the southern parts but with optimum humidity*. I. ricinus* is common in territories from England and Ireland in the west to the Volga - in the east. This species reaches the highest abundance in the zone of deciduous forests, but also could inhibit the territories in the north with mixed and coniferous forests of Scandinavia and Karelia and in the middle-taiga forests of Russia (Filippova, 1977). In the humid and maritime climate of countries of Western Europe, *I. ricinus* could inhabit not only in forests but also in open pasture type biotopes with coarse cereal grasses, moorlands, and bushes. In countries of the Mediterranean belt, *I. ricinus* inhabit the moisture-proof and cool forests in river valleys and on the northern slopes of the mountains (Gilot e. a., 1975; Kalteririeder E. a., 1985). The territories of the middle-taiga, southern taiga and broad-leaved forests from the Baltic to the Pacific Ocean, including Manchuria and Japan are inhabited by ticks *I. persulcatus*. Also,

I.persulcatus inhabit the territories of medium and south taiga forests and deciduous-coniferous forests of the south of the Far East, but within these territories, *I. persulcatus* ticks are absent as in marshy areas and on the highly sparse pine forests on sandy soils areas as well. The distribution of *I. persulcatus* ticks to the north is limited by the sum of the temperature of at least 1400-1600 ° during the warm season. In the north parts, they could inhabit only the areas provided with optimal temperature. Thus, in the North *i. persulcatus* ticks do not expand 63 °northern latitude, and in the European part of Russia, the distribution of *I.persulcatus* is limited by to 64 ° northern latitude. The distribution of ticks *I. persulcatus* to the South is limited by humidity deficit, where this species is inhabited in isolated forests, in forest-steppe, Siberia and the coniferous forests of Tien Shan (Korenberg, 1979). The ticks *I. ricinus* and *I. persulcatus* have limited mobility. The maximum distance for larvae of *I. persulcatus* from the hatching site is up to 1.5 m (Levin, 1987). The imago of *I. persulcatus* could only crawl 5-5.7 m, usually, this distance does not exceed five meters toward the direction of the victim smell (Balashov, 1958; Arumova, 1979). The tick distribution mostly is carried out by animals and birds. In a case of larvae and nymphs of *I. persulcatus*, the distance is limited by tens of meters as they parasites mostly on small rodents. Small distances limit the migration of small rodents. However, in a case of parasitizing on rabbits or hedgehogs or other medium-sized animals the distance of tick's migration could achieve a hundred meters. Large animals as moose and birds could transmit the ticks for long distance. Birds are an ecological niche for diseases transmission for a long distance. In literature, it was mentioned that infected *I. ricinus* was taken off from a bird and the transportation of the viruses into the new not endemic areas is a common event(57).

Moreover, in old literature was noted that the birds play a huge role in a tick transmission. In Ukraine for a time period of less than 80 years has populated and reached a high number of *I. ricinus* of the artificial forest plantations “Askania-Nova”, surrounded by large ploughed fields (Emchuk, 1972). Larvae and nymphs of *I. ricinus* was constantly taken off from migratory starlings, thrushes and other species of migratory birds in the Baltic region (Brinck e. a., 1965).

Usually larvae and nymphs feed on an animal 2-6 days and female imago - 6-12 days. The feeding or parasitic time does not exceed o1.4-2% of the total duration of their life cycles. Each of these short feeding periods is divided by many months of life and development of *I. persulcatus* in the forest floor and grass layer. So, *I. persulcatus* ticks during this time behave like typical tillage or plant-dwelling organisms. They constantly lose water for transpiration through the integuments of the body and face the

danger of drying, and a danger of the death from low temperatures (Balashov, 1989). The optimal humidity of environment is an important factor for surviving the ticks of *I. ricinus* and *I. persulcatus* as well. In the environment lack of humidity, hungry ticks *I. persulcatus* and *I. ricinus* usually face with the death (Balashov, 1989). The fed *I. persulcatus* ticks could face the mortality when after the feeding on an animal ticks *I. persulcatus* falls off into habitats not suitable for their further development as treeless meadows and pastures, marshes, etc. However in a case ticks *I. persulcatus* falls off in favorable conditions for them as litter, sod of plants and soil microwaves, the risk of death from drying out or overheating is minimized. Due to the development of the complex of adaptations for seasonal climatic conditions the ticks *I.ricinus* and *I. persulcatus* could survive during the severe winter. And one of such survival mechanisms is a diapause. The fed female imago could not have a diapause. But there are also the difference in ticks, *I. ricinus* ticks have the diapause in all stages of development while *I. persulcatus* could have the diapause only the larvae and nymphs and starving female imago. If the weather conditions are favorable the ticks of all stages and different time period and years of feeding are activated. In a case of not favorable weather condition a significant part of ticks can continue diapause. And after the surviving through the not favorable the activated ticks consist of different stage from larvae till imago and different year of hatching. Thus, for natural foci of tick-borne infections are characterized by extremely complex system of biocenotic bonds. In fact, the natural focus of infection includes several interrelated or partially independent parasitic systems, each of which supports its own natural path of pathogen circulation. Ticks as biological carriers of these pathogens have not only a transmissible role by receiving and transmitting pathogens but could save and transmit the pathogens without the participation of vertebrates within their populations by transovarial and sexual ways.

2.1.1.8 Tick-borne encephalitis in Kazakhstan

The symptoms of TBE or also called Russian spring-summer encephalitis (RSSE) was described in patients from Almaty and Almaty oblast in 1935 by Steblov (58). TBEV was isolated in Kazakhstan for the first time in 1941 by Chumakov. For this isolation, he used the brain from a patient who died on TBE from Almaty oblast (11). In 1947, Linetskaya isolated TBEV for the first time from the tick species *I. persulcatus* using suckling mice which were suggested by Chumakov (59) (23). However, only in 2014, the first molecular biological data from a TBEV strain (Almaarasan) which was collected in 1977 in Almaty Oblast was published in by Lvov (60).

Kazakhstan shares the borders with countries which are endemic for Tick-borne encephalitis virus, e.g., in Russia (18) (19), China (17)(25)(26), Kirgiz republic (27) and Mongolia (28) (29). Further, Kazakhstan has a wide range of climatic and vegetation zones with favorable conditions for different vectors of zoonotic diseases like TBEV (22)(30). Characteristical TBEV endemic zones in Kazakhstan seem to be mountains (61) and forest-steppe zone like the landscape of Almaty, Almaty Oblast and in East Kazakhstan Oblast. In the South of Kazakhstan, the natural foci of TBEV are mountains Tyan-Shan and foothills of mountains Tyan-Shan. These foci do not have any connection with the West-Siberian group of TBEV foci. In the East of Kazakhstan, the natural foci of TBEV are in forest and forest-steppe zones. These foci located not far from the Tobol-Ishim foci of the West-Siberian group of TBEV foci. Moreover, these foci are adjusted to the Altai foci of TBEV (8).

According to landscape, there are four natural foci of TBEV as the South Kazakhstan foci, forest-steppe and steppe foci, the steppe focus, semi-desert focus (8).

The TBEV South Kazakhstan focus: this focus includes the mountain Tyan-Shan, foothills of mountains Tyan-shan and the South Altay mountains. It characterized as forest-steppe zones at altitude 1500-1700m and meadow and steppe zones till 2700m. The meadow and steppe zones are divided into the low belt with the aspen forest (up to 2100 m), the middle belt with the spruce forest (up to 2300m.) and the high belt with the spruce forest, meadows and steppe. In average, the temperature in summer is +23, and in winter is- 7.4^0C. The annual precipitation is 560 mm. The main vectors of TBEV are *I.persulcatus,* although ticks *I.pavlovsky* also inhabit this focus. Moreover, tick *D.marginatus, D.pictus, and H.punctata* are collected in this area*.* This focus includes Almaty oblast (area) and the East Kazakhstan oblast (area). These oblasts (areas) are recognized as endemic oblasts for TBE (61) (8).

Forest-steppe and steppe focus of TBEV. This focus is in the center of Kazakhstan and includes two oblasts (areas) Kostanay and Akmola oblasts (areas). The virus was isolated from a patient with TBE and ticks spp. *Dermacentor in 70th.* Till 2017 this focus was inactive, but since 2017 the cases of TBEV have been registering by authorities. Also, the investigation of this focus is necessary. Mainly two types of ticks prevail *D. marginatus* and *D. pictus* in this focus*.*

The steppe focus. This focus is also located in central Kazakhstan and located in Karaganda oblast. This focus was also recognized in the middle of 70th a focus of TBEV. The virus was isolated from a patient and ticks *spp. Dermacentor.* Currently, this focus is inactive. However, some TBEV cases started to register since 2017.

Semi-desert focus. This focus was found occasionally during the research. It is not typical for TBEV landscape because the sand desert Saryesik Atyrau occupies the landscape of this focus. The investigation of ticks from this focus *revealed positive ticks D. niveus, Hyaloma asiaticum.* This focus is not active. The foci with forest landscapes have the highest activity whereas the foci with the steppe and desert landscapes have the lowest activity, mostly is not active(62). Despite the ticks*, I. persulcatus* is the main vector of TBEV in Kazakhstan, it was revealed that ticks of the other 12 species were positive for TBEV. These ticks species are *I. pavlovskyi, I. trianguliceps, I. gexagonus, I. gibbosus, Haemophisalis concina, H. japonica, H. inermis, Dermacentor marginatus, D. silvarum, D. reticulatus, D.nuttali* (63)*.* However, this data should be rechecked as in 70th and 80th the diagnostics approaches were different and mainly methods based on antigen detection were used as direct hemagglutination reaction and immunofluorescence test (63) (63). TBEV could have a cross-reaction within the flaviviruses (64). Moreover, according to the recent study, the circulation of other flaviviruses as West Nile virus could be on the territory of Kazakhstan and adjusted territories (6).

Therefore, the investigation of this foci is under a big necessity.

During the observation was noted that the large ungulate animals are the host as for imago *I.persulcatus* and larvae and nymphs of *I.persulcatus* as well(61).

In Kazakhstan two oblasts (areas) are endemic for TBE as Almaty and the East Kazakhstan oblasts (areas) as well (*figure 2).*

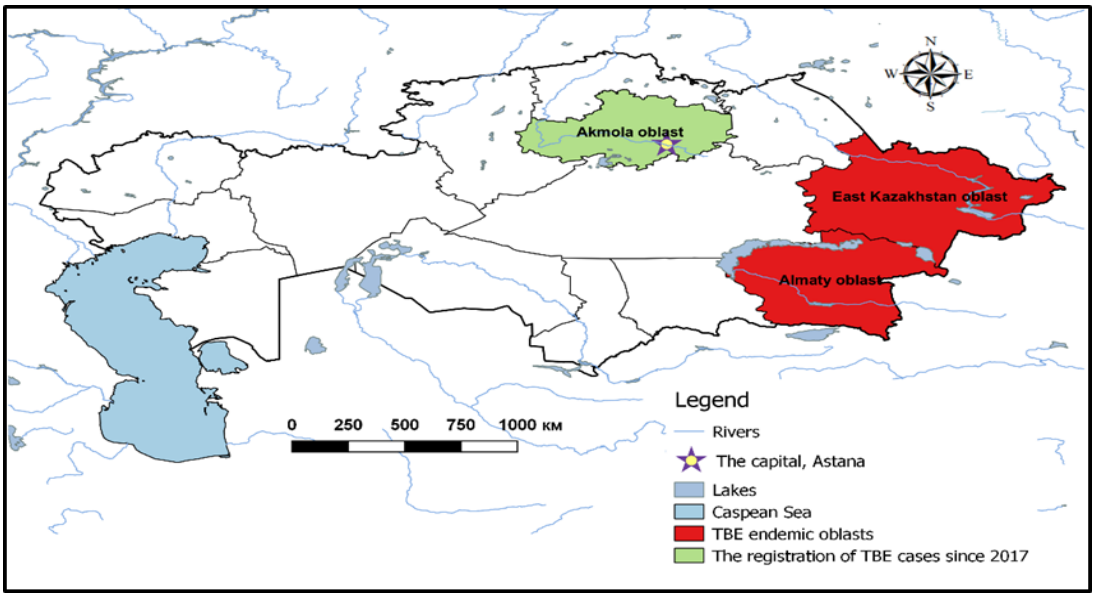


Figure 2. The endemic oblasts (areas) for Tick-Borne encephalitis in Kazakhstan

TBE cases in Kazakhstan are on a constant high level with reported incidence rate to be 0.16/100.000 up to 0.32/100.000. Approximately 50 cases are registered annually. Refer to the annual report of the Scientific Practical Center of Sanitary Epidemiological Expertise and Monitoring during the last three years (2016-2018) 126 cases of tick-borne encephalitis were registered. Moreover, according to this report since 2018 TBE cases have been started to register in the North and the center of Kazakhstan.

Despite this increased awareness, there is still a lack of data regarding the circulating TBEV strains and their molecular biological data. The data over the TBE cases mainly formed from the medical facilities reports based on clinical and epidemiological peculiarities. Despite the annual reports on tick bites and reported TBE incidence there is only a few compressed data are available on the infection rate of TBEV in ticks in endemic areas, so far.

Moreover, since 2017 in TBEV foci, previously inactive, the TBE cases have been started to register. Most of the cases could remain truly undiagnosed as only serological methods of investigation are available in laboratories and serological approaches based on commercial kits with unknown specificity. Moreover, it is a big debate whether it is true TBEV in again active TBEV foci or another flavivirus. The data over the actual condition of natural foci of TBEV needs to be updated. The aim of this thesis to discover some posed questions and make the basis for future studies in this field. However, the most substantial part of this project that it will bring knowledge over TBEV and improve as surveillance system over TBEV and also diagnostic approaches of this infection in medical facilities.

2.1.2 Literature review. Congo-Crimean Hemorrhagic Fever (CCHF)

Crimean-Congo Hemorrhagic Fever is an infection caused by an arthropod-borne virus, the Crimean-Congo Hemorrhagic Fever. This arboviral infection has a specific clinical manifestation such as hemorrhagic fever with a high fatality rate up to 30%(65).

2.1.2.1 History of CCHF

CCHF has been known for centuries. The first mention about the disease was published as early as the XII century (1100) in Persia in work "The Treasure of Khorezmshah" by the Dzhurzhani. He described a disease that was close or identical to CCHF and had a clinical manifestation with abundant nasal, gastrointestinal, pulmonary bleeding and gingival bleeding as well. The disease was associated with a tick bite parasitic on a black Griffin. The existence of CCHF in Central Asia in remote times was

pointed out by many modern authors. For centuries, this disease has been known in Tajikistan and Uzbekistan under the various names. Historically the name of the disease is originating from cases occurring in summer 1942 in hospitals of the occupied Crimea. 18 cases of peculiar gastric bleeding observed in Stalinbad (currently Dushanbe) were described in 1944. The respective disease was called acute infectious capillary toxicosis, by its clinical and epidemiological characteristics, and so fully matched the clinical symptoms of CCHF. Afterward, in 1944-1945 an outbreak of severe febrile illness with severe hemorrhagic syndrome occurred in the Crimea within approximately 200 soldiers. The disease was characterized by a lesion of the vascular system and was initially designated as “acute infectious capillary toxicosis” and thereafter named by. Chumakov as “Crimean hemorrhagic fever” (66)

Many species of a laboratory, domestic, and wild animals (including adult white mice, guinea pigs, monkeys, and cats) were resistant to infection with virus-containing materials. However, in 1945 Chumakov et al. established the viral etiology of CCHF by the reproduction of this disease in psychiatric patients who needed pyrogenic therapy. Later, it has been shown that the suspensions of *Hyalomma plumbeum* ticks and the blood of patients with CCHF contained a viral pathogen detecting even after passing through small-pore filters and caused the disease with a clinical manifestetion of CCHF. In 1967, it was decided to use the method of intracerebral infection of newborn white mice - a model that at that time was extremely effectively used in foreign laboratories to isolate many arboviruses. Nine strains of CCHF virus were identified: a strain “Drozdov" from a patient of Astrakhan Oblast, seven from patients from Rostov oblast and one from a patient from Samarkand oblast (currently Uzbekistan). The evidence of the etiology of CCHF was completed by the consistent detection of seroconversion of specific antibodies in patients with a typical picture of the disease and the regular release of this virus from their blood during the acute phase of the disease. Serological discrimination to 20 other arboviruses was shown in 1968 the CCHFV strain "Drozdov" was transferred to the arbovirus laboratory (YARU) at Yale University, USA, to identify the similarity between the strain Drozdov (a prototype of Crimean Hemorrhagic Fever Virus) and strain Congo. This strain was isolated S. Courtois in 1969, in the medical laboratory of Stanleyville (Zaire, Belgian Congo) from the blood of a sick child(67).

Taxonomic status was established in 1969 when the V3011 strain of the Congo virus was found in Uganda. As a compromise between the "unofficial" historical priority and the "official" registration criterion, Casals and co-authors (1970), suggested the general name of the Crimean-Congo Hemorrhagic Fever Virus, which was adopted in the

international scientific community. The substantiation of the etiological unity of this infection in the endemic regions of Europe, Asia, and Africa. Moreover, the most valuable is the determination of the biological and morphological characteristics of the virus. Hence this finding allowed establishing its belonging to the family *Bunyaviridae.*

2.1.2.2 Microbiology of CCHF

Recently the taxonomy of CCHFV was changed. Crimean Congo Hemorrhagic Fever virus now belongs to the genus *Orthonairovirus*, family *Nairoviridae*, order *Bunyavirales* (ICTV taxonomy.2017 [released 12 March 2018]) https://talk.ictvonline.org/taxonomy). The genome of CCHFV contains three segments RNA with the large (L), the Medium (M) segment (~5.5 kb) and Small (S) segment (~1.6 kb). The L segment (12.5 kb) encodes a Large (L) protein and contains the multiple and highly conserved domains as the RNA-depended RNA polymerase, an Ovarian Tumor (OUT)-like cysteine protease domain and a zinc-finger domain (68). The size of L protein in comparison to other segmented negative-stranded viruses is ~450 kDa twice prominent. Also, this protein plays a significant role in virus transcription and counteracts the possible immune response of a host (69) as on the N terminus of L protein located the ovarian tumor domain. This domain is responsible for suppressing the immune system of a host cell (70). The medium (M) segment encodes a unique polyprotein. It is processed into the polyprotein in the endoplasmic reticulum. Later, this polyprotein is cleaved into pre-GN and pre Gc precursors. These pre-Gn and Pre Gc precursors require the activity of cellular serine and endo-proteases as SKI1 or SIP and furin for maturing into Gn and Gc proteins. These proteins are crucial for immunity and pathogenicity.(71). CCHFV could interact with host cell surface receptors only via GN and GC glycoproteins. Moreover, the GN and GC glycoproteins are the primary target for neutralizing antibodies. This ability could be used in a vaccine creating, and treatment as some studies reported that convalescent human sera could release the protective mechanism in an acute phase of the disease (72) (73)(74). The small (S) segment encodes the nucleocapsid protein that is responsible for encapsulation (75). Phylogenetic analyses of CCHFV by comparing S segments shows seven geographically separate genetic clades due to the IV clade is divided into two different clades Asia 1 and Asia 2(76). Whereas by M segment this clustering is into six clades (77).

Table 1. The phylogeny grouping of CCHFV by S segments

Clades	CCHFv groups
Clade 1	1. .West –Africa
Clade 2	2. Central Africa
Clade 3	3. South Africa and West-Africa
Clade 4	Middle East and Asia 4. Asia 1 5. Asia 2
Clade 5	6. Europe Clade
Clade 6	7. Greece

However, according to Hewson et al., 2004 the phylogeny grouping by M segment is different. Also, it was established that segmented RNAs is more vulnerable to reassortment if (78). This evidence was also observed during the project study.

2.1.2.3 Clinical presence of CCHF

It is still not clear whereas the geographical distribution could affect the virulence in human (79), but some studies show that strain AP92 from Greece had asymptomatic and mild clinical manifestation (80). Usually, the clinical manifestation is developing in four stages: The incubation, the pre-hemorrhagic phase, the hemorrhagic phase, and the convalescence(81). The severe cases could progress into a clinical presence with disseminated intravascular coagulation(DIC), shock and heavy bleeding (75). The reasons for the severity of CCHF clinical presence is still under the question. Moreover, according to some studies the late or the absence of antibody response (82) (83) is a predictor of fatality or severe clinical presence of CCHF. The fatality rate reaches 30%(65). However, many studies show that clinical presence of CCHF could be presented only with not specific symptoms as fever in a certain proportion of patients in endemic CCHF region and it is usually remained undiagnosed by physicians (5)(2)(84).

Despite the absence of the generally accepted classification on the clinical manifestation of Crimean-Congo Hemorrhagic Fever, most authors use the generally accepted scheme of distinguishing the clinical presence into severe, moderate and mild forms (Clinical recommendation for CCHF, 2014, Russian Federation) (85). The description of Crimean-Congo Hemorrhagic Fever by their severity (66)

A mild form of Crimean-Congo Hemorrhagic Fever is observed in 9.5% of cases. This form of the disease is characterized by short-term sub febrile fever. The symptoms of intoxication are mild and are manifested by drowsiness, lack of appetite, general

malaise and moderate bradycardia as well. The hemorrhagic syndrome is manifested by an abnormal, rapidly disappearing rush. The nose bleeding is observed rarely, and usually, the nosebleed is not abundant. The Blood picture is characterized by moderate thrombocytopenia, leucocytes level is within the normal range, but in some cases a slight leukopenia. The outcome of the disease is favorable.

The moderate form of Crimean Congo Hemorrhagic Fever was observed in 26.2% of cases. Fever is lasted 4-5 days approximately and reached high numbers. Symptoms of intoxication are expressed: weakness, exhaustion, drowsiness, blood pressure decreasing, weakness and bradycardia as well. However, some patients suffer from tachycardia. A hemorrhagic syndrome is manifested by gingival bleeding, profuse hemorrhagic eruption. The rush is increasing. Also, the hemorrhages are presented at injection sites. Bleeding is abundant and often repeated as well. In the blood picture, such changes could be observed: leukopenia, anemia, thrombocytopenia. The outcome of the disease usually is favorable.

The severe form of Crimean-Congo Hemorrhagic Fever was observed in 64.3% of all cases. The disease is characterized by acute development. The initial period is shorter, not more two days and lasting on average 1-2 days. Approximately temperature is reached 39–40 ° C. The symptoms of intoxication are severe and includes nervous system lesions. Several patients have delirium, hallucinations, loss of consciousness. Also, meningism and focal neurological signs could be observed. Most patients have tachycardia, hypotension, muffled heart tones, and in some cases, an arrhythmia is observed as well. A severe course of the Crimean-Congo hemorrhagic fever is characterized by abundant, repeated bleeding (nasal, gastrointestinal, pulmonary, uterine), simultaneously from several organs. Blood loss could reach one-two litters. In the blood picture the number of erythrocytes decreased (2.0 * 1012 / l), leukopenia (2.0 * 109 / l and below), thrombocytopenia reached 60.0 * 109 / l, and in some cases the number platelet count decreased to 7.0 * 109 / l - 4.0 * 109 / l. Complications are often developed in a severe form of Crimean Congo Hemorrhagic Fever. 50% of all severe cases of Crimean-Congo Hemorrhagic Fever was fatal. Recovery usually is slow.

Despite such dividing of Crimean-Congo Hemorrhagic Fever into mild, moderate and severe forms In Kazakhstan the updated CCHF classification of Leshinskaya 1967(85)

is used. This is also recommended by the Health Care Ministry of Kazakhstan. According to the classification (Leshinskaya, 1967), Crimean-Congo hemorrhagic fever is divided into Crimean-Congo hemorrhagic fever with the hemorrhagic syndrome and Crimea-Congo hemorrhagic fever without the hemorrhagic syndrome.

***Classification of CCHF by Leshinskaya,1967*.**

I. ***CCHF with hemorrhagic syndrome***:

1. A severe form of Crimean-Congo hemorrhagic fever:

a) Without large hematomas on the skin and mucosal membranes and bleeding in cavities (epistaxis, hematemesis, melena, and intra-abdominal bleeding)

b) With large hematomas on the skin and mucosal membranes and bleeding in cavities (epistaxis, hematemesis, melena, and intra-abdominal bleeding

2*. Moderate form:*

a) Without hematomas on the skin and mucosal membranes and bleeding in cavities (epistaxis, hematemesis, melena, and intra-abdominal bleeding

b) With hematomas on the skin and mucosal membranes and bleeding in cavities (epistaxis, hematemesis, melena, and intra-abdominal bleeding

3. *Mild form*.

II. CCHF without hemorrhagic syndrome:

1. *The moderate form;*

2. *Mild form.*

The severity of CCHF was determined by the following criteria: The intensity of intoxication, the level of hemorrhages, and a blood picture. Currently it was proved that the severity of CCHF and outcome as fatality correlates with the level of liver enzymes as alanine aminotransferase (ALT) ≥150 U/l, aspartate aminotransferase≥200 U/l and activated partial thromboplastin time (aPTT) ≥ 60 sec, fibrinogen ≤110 mg/dL(85)(86), counts of platelets (PLT)≤20×10^9/l, leucocyte counts ≥10×10^9/l(85)(86), counts of platelets (PLT)≤20×10^9/l, leucocyte counts ≥10×10^9/l (85)(86).

Treatment of Crimean-Congo hemorrhagic fever

The treatment of CCHF based on antiviral and supportive therapy. Currently, among the plurality of antiviral medicines, only Ribavirin is approved by WHO. The efficacy of ribavirin against CCHFV is still unclear, but some authors explain it is by its immunomodulatory effect (79) www.WHO.org, www.cdc.gov .

Some observational studies show the efficacy of Ribavirin, even most of the studies based on oral ribavirin even WHO recommends the intravenous form of ribavirin (87). However, the question of Ribavirin efficacy as oral and intravenous forms as well is still under big debates (88) as the only observational study was conducted and any randomized trials were conducted. Therefore, such observations could be biased (89). Many authors suggest including Interferon for the treatment of CCHF. The viral nature of the disease, pathogenetic validity and the proven efficacy of the use of Interferon type

1 in many viral infections provide the basis for the inclusion of Interferon in the combined therapy of CCHF. Interferon type 1 has anti-viral, immunomodulatory and anti-proliferative properties. It is now established that the protective antiviral effect is realized mainly through the activation of the immune system(66) and some authors mention its beneficial effect against CCHFV. The conducted experiment demonstrated that Interferon alfa and beta induces the releasing the MX (MX1 in mice and MXA in humans) proteins as well. During the same experiment, it was proved that MXA proteins have antiviral activity against CCHFV(90). (91). Later was proved that interferon alfa induces the expression of interferon genes. This expression could be necessary as a protective factor against CCHFV infection. CCHFV infected the pools of STAT1 Ko and WT mice. It was demonstrated that the lack of interferon genes expression was led to more rapidly the virus dissemination and fatal outcome.(92).

The efficacy of interferon treatment is still taking the attention of scientist

In some countries, also in Kazakhstan, the CCHF convalescent sera transfusions is widely applied in endemic regions for CCHF treatment. This method was suggested as early as in 1945. Despite positive outcomes there is still big question upon the efficacy of this method as not enough data is performed (88). However, in Bulgaria is a commercial specific human immunoglobulin "CCHF-venin" available (74). The supportive therapy as an infusion of blood components, transfusion of frozen blood plasma, infusion of fluids, respiratory support required as needed and should be considered in every case (93).

2.1.2.4 Vaccines CCHF

Due to the unavailability of effective vaccination, specific treatment and high mortality rate. Crimean-Congo hemorrhagic fever virus is included in biosafety level hazard risk. Moreover, the climate change and expanding the territories affected by the main vector of Crimean-Congo hemorrhagic fever virus ticks *Hyaloma marginatum marginatum*, globalization and international tourism to countries endemic for Crimean-Congo Hemorrhagic Fever as well(94). There is a high demand for an effective vaccine. Currently, only one vaccine against CCHF is known; this vaccine was produced and only licensed in Bulgaria. This vaccine was manufactured by using the Crimean-Congo hemorrhagic fever virus strain V42/81, an isolate from a patient suffered from CCHF in 1981 and inactivated by chloroform, heating at 58^0C and adsorbed on $Al(OH)_3$ (Vasilchenko S., 1976) (95) (96). However, this only existing vaccine against the Crimean-Congo hemorrhagic fever; the efficacy of it is under the big debates as the

efficacy data is still unavailable (94). So, the new vaccine platforms are needed. Recently several studies on developing vaccines against the Crimean-Congo Hemorrhagic fever have shown their confidence in pre-clinical trials (www.cchfvaccine.eu). The different approaches were reported as DNA vaccine with an expression of determined CCHFV genes or the vaccines with viral or plant vector.(97) (98) (99) (100) (101) (94) (102).

2.1.2.5 Biosafety containment of CCHF

Biosafety containment of CCHF Due to high infectivity and unavailability of a protective vaccine, the absence of effective treatment CCHF is included in a group 4 of hazard risk and requires Biosafety Level 3- 4 laboratories to handle with positive CCFHV material or material suspicious for CCHFV according to international guidelines(103)(44) . Observational studies demonstrated the incidents of CCHFV laboratory-acquired infections and have a high risk for nosocomial infection (104) (43). Moreover, according to these recommendations before the manipulation with CCHF viral RNA, the samples contained the infected material should be inactivated (43). For serological methods the heating for 30 min at 560C. In experiments conducted in Africa for clarifying and analyzing the appropriate temperature for virus inactivation was proved that heating at 560 C inactivate sample (43)(105) In a case of molecular methods of investigation in an experimental study, the high efficacy of virus inactivation demonstrates AVL buffer contains a chaotropic salt (Qiagen, Hilden, Germany)(106) (107) and TRIsolR LS reagent is a monophasic solution of phenol and guanidinidine isothiocyanate (Invitrogen corp.). Qiagen kits for virus RNA extraction includes this buffer, and this buffer does not inhibit the PCR (Qiagen mini-viral kit instructions). The ability of virus inactivation was proved by the absence of virus growth and plaques formation. These reagents are highly effective to inactivate the viruses of different four genera as bunyaviruses, flaviviruses, filoviruses, and alphaviruses (41).

2.1.2.6 Diagnostics of CCHF

Timely started diagnostics and treatment are essential for good patient management and improving the ability of preventive measurements. Recent years several methods of diagnostics are developed and established. It could be divided into two big groups as molecular biological methods and serological methods of investigation as well.

Molecular methods of investigation.

The modern methods of molecular investigation is aimed for the rapid identification CCHFV in a field material as ticks and clinical specimens (108).

However, the crucial point for the investigation of clinical specimens by molecular methods of investigation requires the exact time of blood withdraw approximately the within first five-six days after the disease onset.

In 2005 for the first time was presented the method of one step real-time PCR assays for identification and quantification of viral RNA in a short time that is necessary for diagnostics of rapid developing CCHFV infection (109). So far, currently existing primers are designed for all lineages of CCHFV, as the highly conserved region of S segment was the target for this purpose. The viral RNA of 16 unique strains of viruses cover five lineages of CCHFV; these viruses previously propagated in vitro in a high biosafety containment. Remain two strains were synthesized according to the sequences in GenBank. These strains are AP92 from the lineage Europe2 (access number DQ 211638 and DAK8194) and a strain from the lineage Africa 1(access number is U88411)(106).

However, the assays are suitable only for those sequences available in GenBank. Moreover, it is crucial to design primers as new data on sequences are available in GenBank(6). “novel rapid RT-qPCR for detection of the various genotypes of CCHF”(110), or “Syber Green based one-step rRT-PCR”(111),

As CCHF is mostly widespread in low- and middle-income countries, more rapid, cheaper assays and assays not demanding upon expensive machines were successfully developed in the recent years such as low density, field-applicable microarray CCHFV(112) , (113). Another functional assay in the field in remote rural areas are Loop-mediated isothermal amplification and colorimetric nucleic acid assays for CCFHV detection developed (114) (114) (115).

Serological methods

Serological methods of investigation play a tremendous role in CCHF diagnostics. They are based on the detection of IgM and IgG antibodies against the virus or detecting the virus antigen. The best time for blood withdraws for serological investigation for antibodies against CCHFV is after fifth day illness. As with all viral infections, the diagnostic of CCHFV infections should be done with in-depth consideration of day of illness, possible virus circulation and time of antibodies release (108), as the virus could be detected within the first six days after the disease onset (116). The antibody detection in blood of sick patients not only identify the antibodies

against the pathogen, but also an ominous sign of the disease severity (95). It was noted that among the survivors, antibodies appeared in the early phase of diseases (116).

The best commercial kits for detecting antibodies are IFA, Euroimmun (Luebeck, Germany) and IgM and IgG n ELISA Vector-Best (Novosibirsk, Russia) and are recommend in geographic regions close to Kazakhstan (117). (www.canada.ca).

2.1.2.7 Epidemiology and vectors of CCHFV

CCHF can develop in a severe disease only in humans(75). Despite the developing of antibody response and viremia, the signs of CCHF never appear in animals, except for newborn mice (118)(67)(69). The infected animals play a substantial epidemiological factor to be infected by CCHF as they do not have signs of the disease. Humans can be infected through tick bites, contact with infected blood and tissue of animals, consumption of raw milk (119). Moreover, often nosocomial infections are happening, when medical personnel and members of family contact with biological fluids. The CCHF virus transmission through sexual contacts was described in Russia(120). However, tick plays a considerable role in virus transmission of CCHFV and circulating in nature. The virus distribution reflects the distribution of the main vector of CCHF, tick *spp. Hyaloma* (121)(122). However, the tick spp. *Rhipicephalus*, *Dermacentor*, *Haemaphisalis* could be vectors for CCHFV (122). The ecology of the ticks *spp. Hyaloma* showed that the main hosts of its larvae and nymphs are birds that feed on the earth, mainly rooks. The imago of tick *spp. Hyaloma* parasite on cattle, horses, sheeps and goats. Animals (cows, goats, sheep, hares, hedgehogs) could be a reservoir of CCHF virus. The infected animal the viremia could infect parazatiting on them ticks. The features of the structure of tick *spp. Hyaloma* could explain that these species are adaptive for dry and hot weather because of the structure of cuticle and Haller's organ, the glands of Haller's organs providing good moisturizing(123). Also, it could explain the tick activity from April till the end of July and distribution of these species of ticks in the Southern part of Kazakhstan, where the weather temperature in summer achieves 50 0 C(124) and it explains that these species of ticks circulate in Oriental, Palearctic and several parts of Sub-Saharan Africa (122).

According to an epidemiological study based on statistical estimation, five countries (Turkey, Iran, Afghanistan, Tajikistan, and Pakistan) have the highest evidence of CCHF presence.

Moreover, under the high risk the countries of Central Asia, South-west of the Russian Federation, the regions of Sub-Saharan Africa and Central Africa. The CCHF presence could be active for such countries as Syria, Iran, Iraq, Romania, Moldova, and Ukraine. Moreover, the CCHFV affected territories are expanding due to climate change, increasing of temperature level and decrease in precipitation. All these weather conditions create favorable conditions for tick spp. Hyaloma (the main vector of CCHFV) surviving in case of tick transporting by birds(94)(125)

2.1.2.8 Crimean-Congo Hemorrhagic Fever in Kazakhstan

Geographically Kazakhstan consist of 14 oblasts (areas). Three oblasts are endemic for Congo-Crimean Hemorrhagic Fever. These oblasts are Kyzylorda oblast, Turkestan oblast (the former The South Kazakhstan oblast) and Jambyl oblast (see figure 3). These oblasts share the border with the endemic for Crimean-Congo Hemorrhagic Fever country as Uzbekistan and Turkmenistan where were several outbreaks of Crimean-Congo Hemorrhagic Fever were registered during the Soviet Union Era (67) and the evidence of Crimean-Congo Hemorrhagic Fever presence is also reported nowadays, however the lack of data is available (126).

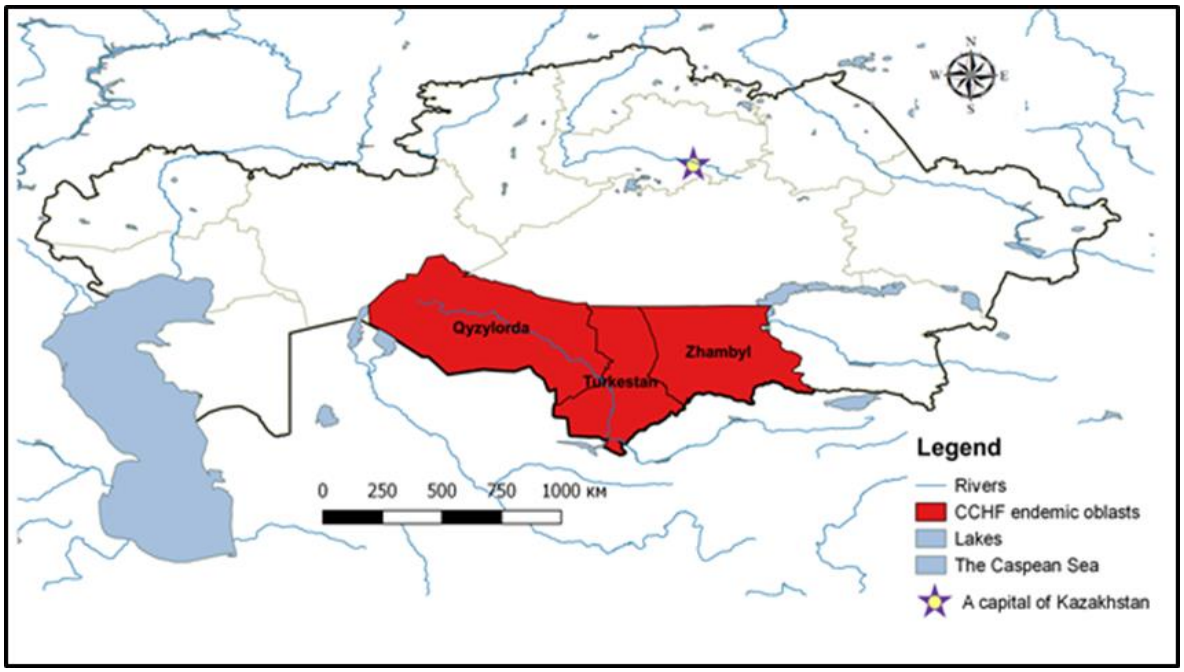


Figure 3. CCHF endemic oblasts (areas) in Kazakhstan

According to Dobritsa P.G, 1965, 1975 the first cases of CCHF were registered in 1948 in the South of Kazakhstan oblast, later the clinical cases of CCHF were registered in Kyzylorda oblast. And only in 1982 in Jambyl oblast a natural focus of CCHFV was identified by the scientific workers of Kazakh Scientific Institute of Epidemiology and microbiology of infectious diseases after the CCHF outbreak. However, this disease was known on the territory of Kazakhstan since ancient time and named as “Kokala” in translation from Kazakh language is “colorful body” because of hemorrhagic syndrome (Genis et al., 1971). This infection was well known among the population of Central Asia. In Uzbekistan, it was named as “kara-halak”, that means a black death, in Tadjikistan (Kolachev,1945; Blyaher et al., 1971) CCHF was known under the name “Hungibta” or “Hunimuni” and it means “bleeding from the nose”(Aristova V.A., et al.,1973;). In 1944 was described in Central Asia by Sipovskii V.V.. He investigated the pathological material of people who died from the diseases with hemorrhagic syndrome. Later the same outbreak of fever with serious hemorrhagic syndrome was in Uzbekistan, Tajikistan and Turkmenistan. In 1947 the fever with serious hemorrhagic syndrome got the name “Central Asian hemorrhagic Fever”. Central Asian Hemorrhagic Fever as separated nosological form till 1967. In 1967 by Leshinskaya and Chumakkov was proved that Central Asian hemorrhagic Fever and Crimean Congo Hemorrhagic Fever are the same disease (Leshinskaya, 1967).

The clinical presence of Crimean-Congo hemorrhagic Fever in Kazakhstan doesn’t have any different features. Crimean Congo Hemorrhagic fever is developing through the incubation that is usually lasting from three to seven days (19) and following by the pre-hemorrhagic phase. But according to some observations made on territory of Central Asia and the South west parts of Russia there are some differences. The incubation time is lasting for 7-12 days according to Kolachev A.A. (Kolachev et al., 1949), by the data of Leshinskaya it could be 3-12 days (Leshinskaya,1967), and by Lazarev V.N. it is lasting 1-14 days. But in 94.2% of patients the incubation time period does not exceed 10 days, 72.1% patients had two-four days of incubation period (Lazarev V.N. Crimean Hemorrhagic Fever in Rostov Oblast, thesis, Moscow, 1973).

Annually 11 cases of Crimean-Congo Hemorrhagic Fever are registered in endemic oblasts (areas) of Kazakhstan(127). Usually, the CCHF cases are registered from April till September during the tick activity. Approximately up to 37.8% of all registered cases occurred in May and June (128).

According to reports of Kazakh Scientific Center of Quarantine and Zoonotic Diseases, Crimean-Congo Hemorrhagic Fever is characterized by the dispersion of natural foci. Long-term epidemiological observations and the data of virological and serological investigations demonstrates, that vectors of Crimean-Congo Hemorrhagic Fever Virus are different. In the southern (Turkestan oblast and former the South Kazakhstan Oblast) in piedmont areas, the foci of Crimean-Congo Hemorrhagic Fever Virus “occupies” small territories and the main vector is *H. anatolicum. H. anatolicum* is mostly parasitic on livestock. On the livestock from this region, ticks H*. anatolicum*, was found in all its life stages from nymph till imago. The natural foci of Crimean-Congo hemorrhagic Fever located in flood plain areas as the territories adjusted to Syrdaria, the main vector is as *H. detricum and H. asiaticum* could be as well*.* A feature of *H. detricum* is that hares, gophers and other rodents are involved into a feeding process. The most active circulation of Crimean-Congo Hemorrhagic Fever Virus is in deserts Karakum, Moyinkum, Sarykum, Saryesik Atyrau and Rin desert. The main vector of Crimean-Congo Hemorrhagic Fever Virus in these regions is *H.asiaticum.* In these regions the virus circulation is maintained between vectors H.asiaticum and hosts. The hosts are camels, livestock, saigas and rodents. The natural foci of Crimean Congo Hemorrhagic fever in these areas are the most dangerous for human(128) (Abdikarimov et al., 1995). In general, the endemic areas have a continental climate, a very hot in summer with a lack of humidity that is very favorable for ticks *Hyaloma asiaticum, Hyaloma anatolicum, Hyaloma suspense* and *Hyaloma marginatum*. All these ticks are presented in endemic region for Crimean-Congo Hemorrhagic Fever. The density of ticks are high in these areas, more than 1500 imago ticks could be collected from a single animal (127). Also due to different landscape in Kazakhstan, there is a wide range of tick species. The antigen of the virus is also presented in ticks spp. *Dermacentor*, spp. *Rhipecephalus*. The wide variety of ticks that could be a potential vector of Crimean-Congo hemorrhagic Virus observed in Kazakhstan is reflected the research that 27 ticks taxa could be associated with Crimean-Congo Hemorrhagic Fever Virus (Hoogstral, 1979) (12).

In 2015 and 2012 the evidence of CCHFV circulation was also identified in not endemic regions for CCHF in tick spp. *Dermacentor* in Almaty oblast, in ticks*. Rhipicephalus pumilio* in the West Kazakhstan oblast and in ticks *H. asiaticum* and *R. schulzei* in Aktobe oblast (*see figure 4*), hence the findings demonstrates that Crimean-Congo Hemorrhagic Fever virus distribution could be expanded over the officially recognized Crimean Congo Hemorrhagic Fever endemic areas *(see figure 4*). But it

could be explained that the endemic areas for Crimean-Congo Hemorrhagic Fever were established according to officially registered Crimean -Congo hemorrhagic Fever cases that have clinical features as hemorrhages. Nevertheless the clinical cases without hemorrhages or not hemorrhagic forms are usually remain undiagnosed (127). According to Abdikarimov et al., 1995 the not hemorrhagic form of Crimean-Congo Hemorrhagic Fever could be up to 9 %, however this number could be exceeded. Therefore, the question of true distribution of Crimean-Congo hemorrhagic Fever is under the big debates. And the knowledge on the proportion of patients with possible Crimean-Congo Hemorrhagic Fever infection among the patients are suffering from the fever, the true prevalence of Crimean-Congo Hemorrhagic Fever Virus in vectors and the circulation genotype of Crimean-Congo hemorrhagic Fever Virus is necessary. The necessity is important because the knowledge will improve the approaches in diagnostics of Health Care of Kazakhstan and surveillance system over this disease. But due to Kazakhstan is also one of the rapid developing countries in the Central Asia with the increased growth of international business opportunities, tourism and multi-national military deployment in neighboring countries, there are an increased demand for epidemiology and molecular biology of Crimean-Congo Hemorrhagic Fever and other arboviruses(7). The aim of this thesis to discover these questions.

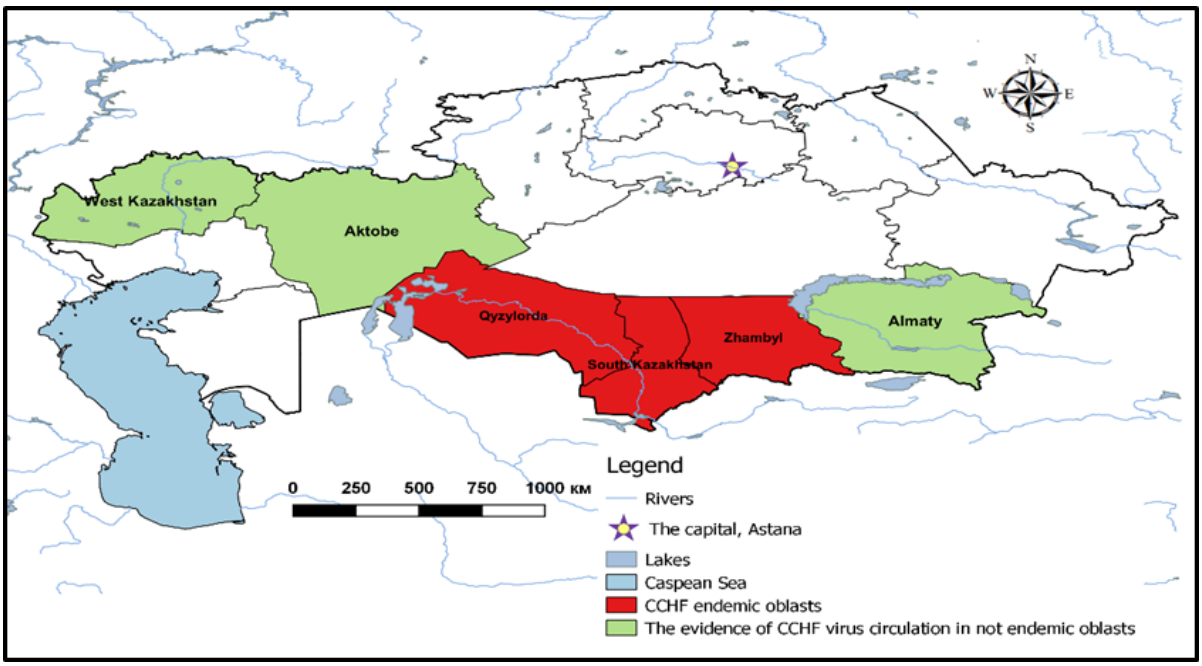


Figure 4. The evidence of CCHFV circulation in not endemic oblasts in Kazakhstan

3 CHAPTER 3

3.1 Rationale and objectives

3.1.1 Rationale of the study

Kazakhstan is one of the countries in Central Asia with the rapid growth of the Economy. The developing and establishment of oil and mining companies make this country interesting for international business. Every year hundreds of employees visit the sites of these manufacturers (www.forum-astana.org). Moreover due to the increasing density of population, developing the local and international tourism and urbanization the risk to be exposed by zoonotic infections as bacterial and viral as well is increasing (4). The awareness of being exposed by dangerous pathogens is reasonable. Kazakhstan has one of the biggest territories in the world, hence it the diverse climate range from the dry and hot in the south and cold with a high level of humidity in the north. The landscapes are desert, semi-desert, mountains, forests. All these environmental factors are favorable for different species of mammals that could be a reservoir for different pathogens. It is known that territory of Kazakhstan is endemic for dangerous zoonotic infections as Plague, Anthrax, Tularemia and zoonotic viral infections as Tick-Borne encephalitis and Crimean-Congo Hemorrhagic Fever. For example, more than 40% of the territory of Kazakhstan is favorable and susceptible to Plague, Anthrax, and Tularemia. Up to 40% of the territory is endemic for Tick-borne encephalitis and Crimean-Congo Hemorrhagic Fever(61). Annually, the incidence of TBE and CCHF are reported. Despite the existence of endemic areas for CCHF and TBEV and incidence of these infections, there is a lack of knowledge on these infections (7). There is no data on the prevalence of CCHFV and TBEV in vectors based on modern molecular methods of investigations in endemic and not endemic areas. In terms of the activation of old foci of TBEV, especially in not typical landscape and vectors, the question of approvement of molecular diagnostics of TBE cases is required. Moreover, there is no data or lack of data on circulating genotype of CCHFV and TBEV. Despite the fact that the possible cross-reactivity with other flaviviruses could be, especially in a country endemic for other flaviviruses as Kazakhstan (6) (148).

The diagnostics of CCHF and TBE even in reference laboratories is based on serological methods of investigations, mainly by ELISA.

The scientists over the world named Kazakhstan as "White spot" (in personal communication) as no new modern data based on molecular methods of investigations are available. Thus, due to the involvement of Kazakhstan in globalization, there is

currently a high demand for the knowledge over these diseases. The knowledge of it is a fundamental basis not only for an accurate diagnostic but also for biosafety and biosecurity measurements as well.

Moreover, the role of these infections as a cause of fever in proportions of patients suffering from the fever in endemic and not endemic areas is still unknown. The few data are only available from the medical hospitals, but the data mostly based on clinical, epidemiological features of the diseases.

Despite the annual incidence of CCHF and TBE, and surveillance system over these diseases in Kazakhstan, the standard operating procedures over the CCHF and TBE diagnostics were not developed.

This thesis aims to improve the exact data on the prevalence of TBEV and CCHFV in vectors of investigated regions.

To understand the prevalence of CCHF and TBE in proportions of patients who suffer from fever in an endemic territory and not endemic territories as well. Also, this thesis is targeted to understand the actual distribution of patients with CCHF. In terms of the authorities of Kazakhstan gives the high rate of lethality, there are big debates over the not diagnosed CCHF cases. Thus, this project study was aimed not only to find out the role of CCHF as a causative pathogen of fever but also to reveal the proportions of patients with CCHF who remain undiagnosed.

One of the principal aims of this thesis is to discover the circulating subtypes of TBEV and CCHFV as well. The molecular data over this disease is a fundamental basis to improve the level of knowledge over the arboviral zoonotic diseases not only in Kazakhstan but also in surrounding territories.

Not a secondary target of this project study was developing and implementing the Standard operational protocols (SOPs) over the laboratory diagnostics (ELISA of IgG and IgM, PCRs,). Also, SOPs over the laboratory work with highly pathogenic viruses and material containing the viruses (Ticks homogenization, Rodent tissue homogenization, Infectious samples inactivation) in Biosafety level 2 laboratories and in the Biosafety level 3 were laboratory developed respectively.

The results of this project study will improve the level of knowledge of the distribution of TBE and CCHF, the surveillance of these infections in Kazakhstan. As CCHF does not have any effective vaccine, and as TBE does not have any specific treatment, the surveillance over these diseases play a substantial role. The knowledge over the circulating strain could help as in the treatment of patients and for biosafety measurements as well. The methods of diagnostics and protocols of molecular

investigations used during the project study could be successfully implemented in diagnostic protocols of target infections and positively influence the diagnostic and surveillance system of the Health Care of Kazakhstan

3.1.2 Objectives of the study

In general, the objective of this study to figure out the prevalence of target arboviruses in vectors and find out their role as a causative agent of fever.

The specific objectives are outlined below:

- To determine the vectors of CCHFV and TBEV
- To calculate the prevalence or minimum infection rate of CCHFV and TBEV in vectors in an endemic and not endemic region as well.
- To detect the circulating genotype of CCHFV and TBEV.
- To determine the proportion of CCHF infection in patients with fever
- To determine the sociodemographic risk factors of being exposed by CCHFV in Kazakhstan
- To determine the epidemiological risk factors of being exposed by CCHFV in Kazakhstan
- To determine the proportion of patients with recent or acute CCHF infection in endemic areas.
- To calculate the proportion of patients with recent or acute CCHF infection in not endemic areas.
- To calculate the proportions of patients with recent TBEV infection in endemic and not endemic areas.
- To work out the standard operational protocols (SOPs) for diagnostics of highly pathogenic viruses as TBEV and CCHFV and to implement SOPs into the
- To update the protocols of the molecular methods of diagnostics to the platforms in Kazakh laboratories and to implement them for diagnostics of TBEV and CCHFV,

4 CHAPTER 4

4.1 Methods

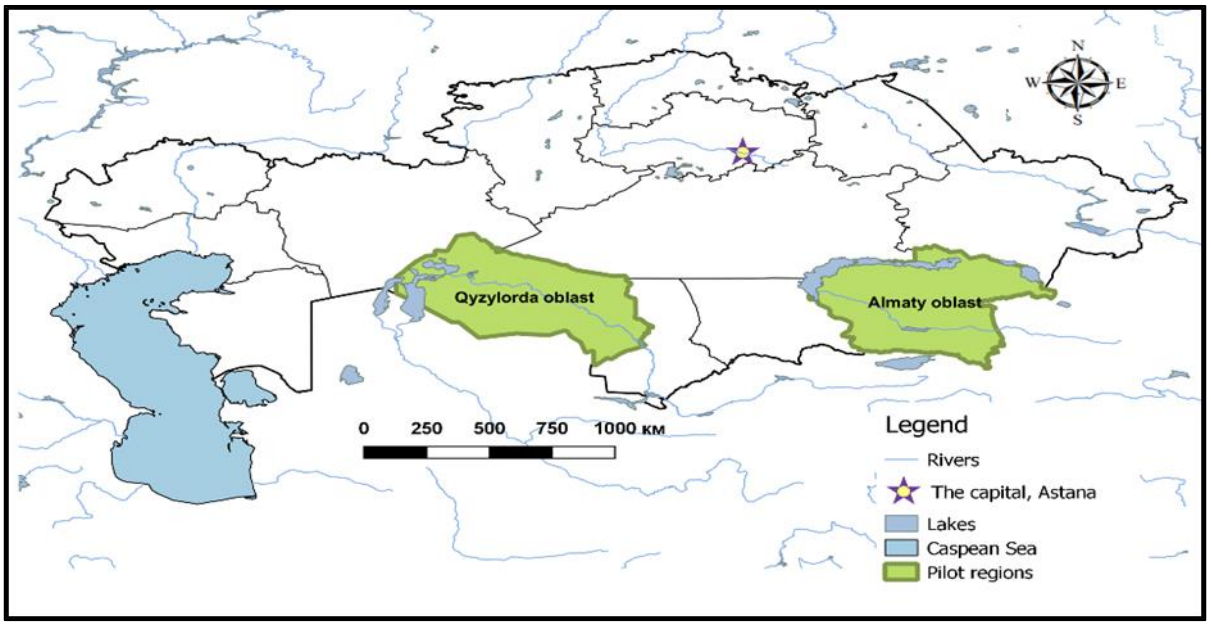
This project study consists of two studies as tick study and serological study as well. During the tick study the ticks from pilot regions have investigated the presence of TBEV and CCHFV, and during the serological study, the sera from patients were investigated for target infections. The methods are in detail outlined below.

4.1.1 Tick study

4.1.1.1 Tick collection

Ticks were collected by flagging at 32 locations of the two selected oblast (Almaty and Kyzylorda oblasts) in May and June in 2015. Sampling was chosen according to routs of tick collection established by entomologists of the Scientific Practical Center for Epidemiological Expertise and Monitoring (SPCEEM). The tick collection was conducted in May-June 2015, during the peak activity of ticks. For each sampling site, GPS data were noted. The collected ticks were morphologically identified and sorted by gender and stage of development. Collected ticks (n=2341) were sorted into pools in laboratory biosafety level 2 of SPCEEM. Each pool contained five imago ticks of the same species.

Samples were collected in Almaty oblast in three districts, Almaty district, Tekeli district and Yenbekshikazakh district at 35 sites, respectively. In Kyzylorda oblast ticks were collected in three districts Zhanakorgan district, Shiely district and Syrdariya districts at 18 sites, respectively.



Kyzylorda oblast **Almaty oblast**

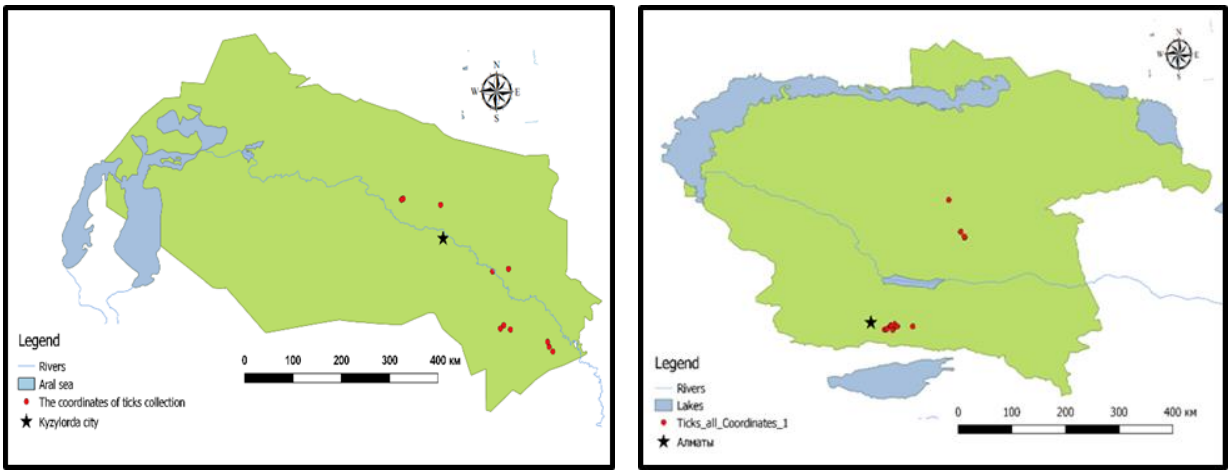


Figure 5. The pilot oblasts (areas) of project study, Almaty and Kyzylorda oblasts

4.1.1.2 Room regime

According to the recommendation of the Laboratory Safety (103), before and after the work in a laboratory at SPCEEM and KMNU, all work surfaces were disinfected by Hypochlorite 0.5%-1% or liquids contain hypochlorite. All manipulations conducted in biosafety cabinet A2. Before the transportation the material, the tubes were placed in the second box and disinfected and only then were transported.

Work with RNAs requires proper disinfection against RNAses. As RNAses are ubiquitous and destroy RNA. For this purpose, we clean all surfaces before the work with RNAse remove liquid.

4.1.1.3 Tick homogenization

During the tick sorting disposable tools were used in terms of cross-contamination. All steps were conducted in biosafety cabinet A2. Before the homogenization, the collected ticks were sorted in pools by gender and stage of development (see 4.1.1.). The ticks were pooled into a previously prepared Eppendorf safe-lock 2.0 tubes containing two ceramic beads and 1 ml medium 199 (suitable for cell cultures) was added. Each pool was homogenized by a Tissue Lyzer (Qiagen, Germany). Before the homogenization, Eppendorf safe-lock 2.0 tubes containing the ticks were placed into the pre-cooled adaptors. The pre-cooled adaptors are necessary, as it prevents increasing the temperature during the shaking and friction. In a case of a not wholly tick homogenization, the procedure was performed again. After the homogenization, the homogenates were used for further RNA extraction or the tubes with homogenates were

placed into the secondary packaging (box). The secondary packaging was disinfected by hypochlorite 0.5%, put into the plastic bag and placed into the freezer at -20. The place was disinfected according to the SOP “Room regime”.

4.1.1.4 RNA extraction

The RNA extraction was conducted with the commercially available "QuiagenRNA mini kit." The kit was used according to the manufacturer’s instructions. RNA extraction was divided into two steps. The first step is Lysis and second the QIAamp viral mini spin procedure.

The Lysis step.

The lysis step was crucial for a sample inactivation. 5.6 µl carrier RNA and 560 µl of AVL buffer were filled into Eppendorf safe-lock tubes 2.0 and 140 µl of a supernatant of the sample (homogenate) was added. This step leads to a sample lysing under the denaturing conditions provided by the AVL buffer supplemented with Carrier RNA. Moreover, the Buffer AVL inhibits RNAses and stabilizes viral RNA. At the same time, the carrier RNA improves binding the viral RNA to QIAamp membrane and reduces the chance of viral RNA degradation.

The QIAamp viral step.

Into the tubes with samples 560µl ethanol (96-100%) was added. Then the sample was shaken carefully for 15 sec (vortex) followed by a short centrifugation step to remove liquid from the top of the tubes. From the lysed probe 630µl was added on the QIAamp Mini Spin Columns without touching the column material and centrifuged at 6000g/8000g for one minute. The eluate was discarded. After the sample was transferred into the QIAamp Mini Spin Columns, washing with buffer AW1 and Buffer AW2 was conducted. AW1 contains the guanidine salt; this salt is a strong denaturant. The RNAses are the most durable proteins and require the guanidine for denaturation (128). So AW1 is used to denature the proteins and denaturized protein could pass through the filters and therefore increase the quality of RNA. This step was following by the centrifuging at 8000 g and the washing step with AW2 buffer. AW 2 Buffer is necessary to remove the guanidine salt (129).

Thus, the washing step with AW1 and AW2 completely removed the residual contaminants. The washing with 500µl of AW1 and then with 500 µ AW2, following by a final centrifuge step at 14000 g. Elution of the RNA was done with by 40 µl of Elution Buffer into sterile PCR clean1.5 ml tubes. The eluting step was conducted by

centrifuging twice for xx minutes at 6000 g 80 µl of eluted RNA was aliquot into 20 µl in 1,5 Eppendorf tubes and stored at -800C.

4.1.1.5 TBEV RT –qPCRs and target E gene conventional PCR

The extracted RNAs from the tick's homogenates (see 4.1.4) were screened for the detection of TBEV RNA. TBEV RNA was detected by reverse transcription (RT)-qPCR (47). 5µl of TBEV RNA was amplified in 25 µl RT-qPCR mixture QuantiTect Virus kit (Qiagen, Germany)) of 0,2 µM of each primer, forward (5'-gggCggTTCTTgTTCTCC), reverse (5'-ACACATCACCTCCTTgTCAgACT) and 0,16 µM of hybridization probe (5'-6FAM-TgAgCCACCATCACCCAgACACA-TMR) with reagents according to the instructions and protocol as it was described previously (47).

Extracted strains of Langat virus (Acc. Number NC_003690) was used as positive control and distilled water as negative control.

To acquire the E-gene the positive samples of the RT qPCR were used to do a conventional RT PCR with primers targeting the E gene region (1632 nt). Briefly, 5 µl of RNA was amplified with 0.2 µM of reverse and forward primers and SuperScript III high fidelity RT PCR System with Platinum Taq DNA Polymerase (Invitrogen) in a final volume of 50 µl (129).

The initial amplification of the cDNA was performed at 50°C for 45 min, then a step of denaturation at 94 ^{0}C for 5 min was carried out. The amplification was conducted for 40 cycles at 94 ^{0} C for 30 sec, 60 ^{0}C during 30 sec and 2 min at 68 ^{0}C. A final extension step at 68 ^{0} C for 10 min was done.

.

Table 2.Primers used for E-gene conventional PCR

Region/District	Forward	Revers
Tekeli samples	947 A+ 947B 5'TCCTCTgCCTggCTCCggTTTATg + 5'TCTTgTgCCTggCTCCggTTTATg	R2579 5-TCTTgTgCCTggCTCCggTTTATg
Talgar samples	947 A+ 947B 5'TCCTCTgCCTggCTCCggTTTATg +5'TCTTgTgCCTggCTCCggTTTATg	R2579 5-TCTTgTgCCTggCTCCggTTTATg
Yenbekshikazakh samples	9947 A+ 947B 5'TCCTCTgCCTggCTCCggTTTATg +5'TCTTgTgCCTggCTCCggTTTATg	R2579 5-TCTTgTgCCTggCTCCggTTTATg

885 5’-ggTTACCgTTgTgTggTTgACC-3’	R2579 5-TCTTgTgCCTggCTCCggTTTATg
885 5’-ggTTACCgTTgTgTggTTgACC-3’	R 2605 5’- TTT CCT TTA Tgg CCg ATg CTA gCg -3’

The amplified products were run in agarose 1.5% gel and visualized by Gel Red® under illumination of ultraviolet light.

4.1.2 Serological study

4.1.2.1 Ethical approval and informed consent administration

The study protocol was reviewed and approved by both ethical committees, the local committee that is located at Kazakh National University named after S.D. Asfendiyarov, in Almaty, Kazakhstan and the ethical committee at the Ludwig-Maximillians Universität, Munich, Germany. Because Kazakhstan is a bilingual country, the informed consent form was translated into two languages Kazakh and Russian. The consent form was given in Kazakh or Russian language; this depended on national originality and ability to think and understand Kazakh or Russian languages. Before enrolment in the study, a volunteer carefully explained the objectives of the study, the anticipated risks, the voluntary nature of participation, about the rights of refusal at any step of study without recrimination to a volunteer.

According to state regulations, adolescents starting from 15 years old are hospitalized in medical facilities for adults. In a case of 15 years old volunteer, the consent form was provided as for a patient and his parents or his guardians as well. (See Annex)

4.1.2.2 Questionnaire

A structured interview-administered questionnaire was provided to every patient (a volunteer). The questionnaire consists of six modules reflecting the sociodemographic, education and work, living and housing, livestock, vector habitat factors, and clinical symptoms. The modules were based on risk factors for target infections identified in the literature review. However, the questionnaire was constructed in terms of risk factors of several diseases that could be a cause of fever of unknown origin as viral and bacterial

as well. The questionnaire was designed several zoonotic infections such as infections with TBEV, CCHFV, Hantaviruses and Rickettsia (See Annex 7).

4.1.2.3 Administration of questionnaires

Trained medical personnel administered the questionnaire. Trained medical personnel entered the collected responses into the questionnaires. Participation in the census was voluntary. A volunteer had the rights do not answer the questions if he felt difficulties in answering. The questionnaires were checked for the completeness by the same medical personnel. The blood withdraw was done after the questionnaire session. Due to Kazakhstan is a bilingual country, the questions, and possible responses were provided in Kazakh and Russian languages

4.1.2.4 Description of variables

- Hospital ID number: a hospital Identification number assigned a volunteer. It is an abbreviation of the city or district.
- Participant ID: A Participant Identification number assigns volunteers. It was a letter and a four-digit number starting in 0001. No volunteer was received more than one number.
- Sex of participant: is gender and categorized as (1) Male and (2) Female.
- Name of Study site: is an area where a study was conducted and categorized as (1) Almaty region, (2) East Kazakhstan, (3) North Kazakhstan, (4) West Kazakhstan and (5) Kyzylorda.
- When were you born: describes a date of birth.
- How old are you: describes the age in years.
- Present marital status: describes the marital status of the volunteer and categorized as (1) Single, (2) Currently married with one spouse, (3) Married with two or more spouse, (4) Not married, living with permanent partner, (5)Separated/divorced, (6) Widowed,(7)Declined to answer,(8)Other.
- Place of birth: describes the country of birth and categorized as (1) Kazakhstan, (2) Kirgizstan, (3) Uzbekistan, (4) Other countries.
- The oblast of Kazakhstan a patient born: describes the oblast (administrative area) where the volunteer was born.
- Living City/Town/Village describes the current address of a volunteer.

- Since when have you been living in this city/Town? Village?: describes the cumulative years of living in the city or town or village and categorized as (1) Always lived in this place, (2) Years
- Have you made any trips from your place of residence within the last month: describes the possible trips and categorized (1) No (2) Yes, specify
- The contact with wild animals: describes the possible contact with the wild animal and categorized as (1) Yes and (2) No
- Bites by ticks, mosquitoes, insects or wild animals within the last month: this describes the possible bites of ticks, mosquitoes or wild animals and categorized as (1) No, (2) Yes, specify.
- The highest level of education: This variable describes the education status of a volunteer and categorized as (1) Still in school, (2) Primary finished, (3) Primary unfinished, (4) Secondary finished, (5) Any higher education, (6) Adult education, (7) Have no formal education, (8) Declined to answer/Don't know.
- The current occupation: this variable describes the current activity and categorized as (1) Pupil, (2) Farmer/Peasant/plants, (3) Farmer/Peasant/animal, (4) Farmer/Peasant/forestry, (5) Keeping the house, (6) Unskilled laborer, (7) Skilled laborer, (8) Local or long distance driver, (9) Administrative or academic professional, (10) Businessman/woman, (11) Nurse / Physician / Clinician / Pharmacist, (12) Unemployed, (13) Declined to answer, (14) Other
- How long have you been working in your current occupation: Describes the cumulative years of working at the current position in years.
- How often do you usually work in the gardens and fields: Describes the frequency of working in the gardens or fields, categorized by (1) Yes, always, (2) Yes, often, (3) Yes, occasionally, (4) Yes, but rarely, (5) No, never
- Ability to read a letter or newspaper easily, with difficulty, or not at all: describes the literacy level of a volunteer and categorized by (1) Easily, (2) With difficulty, (3) Not at all, (4) Declined to answer.
- The total cash income of your household per year: describes the average income of a volunteer
- The number of people regularly eat together in the household: describe the number of family members of a volunteer eat and live together.
- The type of a flat or house: describes the current place of living of a volunteer and categorized by (1) Well-equipped, (2) Poorly equipped city apartment, (3) Private well-equipped house, (4) Poorly equipped house and (5) other

- The storage of bulk products: describes the keeping of products and categorized by (1) In bags, (2) In casks, (3) Other
- The water source: describes from what source a volunteer gets the water and categorized by (1) City water pipe, (2) Rural water pipe, (3) Blow well, (4) River
- The consumption of raw milk or raw milk products from animals: describes the eating of raw milk product and categorized by (1) No, (2) Yes, from which animal
- The types and number of animals in a household: describes the animals in a household and categorized by the type of animal by (1) Cattle and amount.
- Animal's disease: describes if an animal in a household has a disease and categorize by (1) Yes, (2) No
- Animal's disease symptoms: the symptoms are described and categorized by (1) Unusual movement, (2) Respiratory symptoms, (3) Gastroenterological symptoms (4) Lesions, (5) Others
- The death among animals: describes the cases of death among the animals and categorized by (1) Yes and (2) No.
- Contact with death animals: this variable describes the possible contact with dead animals and categorized as (1) Yes and (2) No
- How often direct contact to the following animals was: This variable describes the contact frequency with animals, the frequency estimates from 1 as the Always/daily, 2 - Most of the times, 3 – Rarely and 4 – Never. The determined animals are Cattle, Horse, Goats, Sheep, Pigs, Cats/Dogs, and Poultry.
- The handle with raw meat: This variable describes the manipulation with raw meat as slaughtering, butchering, preparing for cooking and categorized by (1) Yes, always, (2) Yes, most of the times, (3) Yes, but barely, (4) No, never
- How often rats/ mice or bat poop was noticed: This variable describes the frequency of the notice of rats/mice or bat poop. This variable categorized by (1) Always, (2) Most of the times (3) Rarely, (4) Never.
- How often the rats/mice or bats were killed in the house: The variable describes the contact frequency with them and categorized by (1) Always (2) Most of the times (3) Rarely (4) Never.
- The bird nests in the roof: this variable describes the evidence of bird nests on the roof and categorized by (1) Yes, (2) No, (3) Do not know

- Bats live in your house or trees around the house: This variable describes the evidence of bats around the house, and it is categorized by (1) Yes, (2) No, (3) Do not know.
- The location of the house: describes the current placement of the house of a volunteer and categorized by (1) Rural area, (2) Urban area
- The type of the ground around the house: describes the surrounding of the house and categorized by (1) Tarmac, (2) Sand, (3) Dirt
- The look of the vegetation around the residence: This variable describes the possible vegetation around the house and categorized by (1) Dense plantation/forest, (2) Larger grass fields, (3) Occasional bush agricultural fields, (4) Swamp, (5) Lake, (6) Forests, (7) Others, specify.
- The water close to your residence : This variable describes the possible water source and categorized by (1) Puddles after heavy rain, (2) Containers for collecting water, (3) Lake, (4) Steam, (5) Gully in urban area (7) No, never, (8) Don't know
- How many months per year the water around the residence is: this variable describes the cumulative months the quantity of months the water is around the house
- Beginning of clinical symptoms: describes the time of the disease onset and categorized as (1) More than five days ago, (2) Less than five days ago
- The symptoms: describes the clinical symptoms of the disease and categorized as (1) Fever, (2) Headache, (3) Meningism, (4) Weakness of muscles or joints,(5) Muscle pain or recurrent cramps, (6) Pain on swallowing, (7) Joint pain, (8) Stomach/abdominal pain/cramps,(9) Back pain, (10) Earache, (11) Cough, (12) Difficulties in speaking, hearing or seeing, (13) Seizures/epilepsy, (14) Difficulties in breathing, (15) Rapid breathing, (16) Sore throat, (17) Congestion of nose, (18) Enlarged lymph's nodes, (19) Icterus.
- Body temperature: describes the fever level and categorized as (1) Less than 37.5^0C, (2) More than 37.5^0C.
- Duration of high body temperature: describes the cumulative days of fever and categorized as (1) Less than three days and (2) 3 days and more
- Blood pressure: describes the level of blood pressure and categorized as (1) Normal, (2) Hypotension, (3) Hypertension.
- Pulse rate: describes the rate of heartbeats and categorized as (1) Less than 80, (2) 80-100, (3) More than 100
- Skin condition: describes the skin lesions and categorized as (1) Exanthema, (2) Ulceration, (3) Edema, (4) Others

- Stool: describes the changes in stool and categorized as (1) Diarrhea, (2) Blood in stool, (3) Bright stool, (4) Other
- Urine: describes changes in urine and categorized as (1) Blood in urine, (2) Pain on urinating, (3) Dark urine, (4) Low urine volume, (5) Other
- Medications: describes a volunteer took the medicines and categorized as (1) Antipyretics, (2) Antirheumatics, (3) Antibiotics, (4) Other.
- Duration of therapy: describes the cumulative days of therapy
- Similar illness in the family or surroundings: describes the presence of similar symptoms within the family and categorized as (1) Yes, (2) No.

4.1.2.5 Sera collection

Blood was collected from patients on the first day of admission to the target hospital and after 10 or 14 days. The blood was collected from April to October in 2014 and 2015 during the season of tick's activity and from patients according to the criteria of inclusion and exclusion. Into the study, only patients with were older than 15 years old and with fever, more than three days and ear measured were involved. Moreover, patients had to be residents of the investigated area. The patients with confirmed infectious diseases, rheumatological diseases, and rhinitis or without no gained second serum were excluded from the study. The sera were collected in nine target hospitals of Almaty oblast and four hospitals of Kyzylorda oblast. Almaty oblast hospitals were the city hospital of infectious diseases in Almaty city, the regional infectious hospital in Kaskelen city, the regional hospital of infectious disease in Shelek region, in Kapchagay city, in Yessik city, the regional hospitals in Kabanbay, Taldykorgan, Usharal, and Tekeli cities. In Kyzylorda oblast; patients were enrolled in the four regional hospitals of Shiely, Syrdariya, Zhanakorgan, and Kyzylorda districts.

Patients samples were only taken after patients filled in a consent form (In Russian or Kazakh language) before the blood taking was initiated (see Annex 6). Moreover, before the procedure, the patient was informed that participation in the study is entirely voluntary and that they will not receive any financial compensation amount of blood drawn was approximately 20 milliliters covered by coagulation activator as we needed to get the serum without fibrinogen and any blood cells. In maximum, four phlebotomy attempts were made per person. Before the initiation of phlebotomy, the patient was informed about possible risks of procedure of phlebotomy even a small amount of blood was withdrawn. The patient was informed about the possible risk of phlebotomy as ecchymosis, localized skin infection, thrombophlebitis, and dizziness, syncope during or

after the procedure. The procedure was conducted by trained medical personnel in target hospitals. After the blood was withdrawn, the blood was centrifuged, and the serum in the supernatant was transferred into cryotubes and saved at -20^0 C. The scheme of sera investigation (see 4.2.7).

4.1.2.6 Sera inactivation

According to the regulations on biosafety or Laboratory Safety, by the Public Health Agency of Canada (PHAC, www.canada.ca) before the initiation of a serological and molecular investigation, an aliquot of 140 µl of sera was inactivated at 56 ^{0}C for 30 min in a water bath before investigation. The heating was experimentally proved as a safe method of inactivation of possible CCHFV and TBEV viruses (43).

For molecular investigations, additionally to the heating, the AVL buffer was used (Qiagen, Germany which contains a chaotropic salt and is highly effective in inactivation of CCHFV and TBEV (41)(107). During this step into the Eppendorf safe-lock tubes, 2.0 filled with 5.6 µl carrier RNA and 560 µl of AVL buffer were filled into Eppendorf safe-lock tubes 2.0 and. Then 140 µl of the supernatant of the sample (homogenates or serum) was added. This step leads to a sample lysing under the denaturing conditions provided by the AVL buffer supplemented with Carrier RNA. Moreover, the Buffer AVL inhibits RNAses and stabilizes viral RNA. At the same time, the carrier RNA improves binding the viral RNA to QIAamp membrane and reduces the chance of viral RNA degradation. The sera inactivation by heating for serological methods of investigation or lysis step by AVL buffer for further molecular methods of investigation were conducted at SPCEEM, Almaty, according to all biosafety regulations and recommendations.

4.1.2.7 RNAs extraction

The RNA extraction was conducted with the commercially available "Quiagen RNA mini kit." RNA extraction was divided into two steps. The first step is Lysis was conducted at SPCEEM (see 4.2.5) and second the QIAamp viral mini spin procedure. The kit was used according to the manufacturer’s instructions

The QIAamp viral step was conducted at KNMU (see 4.1.4).

4.1.2.8 The Enzyme linked immunosorbent assay (ELISA)

All sera samples were screened by ELISA using commercial ELISA kits (Vector best, Novosibirsk, Russia) for detecting antibodies IgG and IgM against CCHFV and

commercial kit for the detecting antibodies IgG and IgM against TBEV (Euroimmun, Germany) according to manufacturer's instructions.

According to the scheme of investigation, the paired sera that were screened for the presence of IgG and IgM antibodies against TBEV and CCHFV.

In detail the first and the second sera was taken from the same patient in time differences 7-10 days. Firstly the second serum was screened for IgG. If the second serum of the paired human serum was positive for IgG, it was further investigated in the same run with first serum. In a case the first serum was negative for IgG, it was further investigated for IgM against TBEV and CCHFV. The first serum negative for IgM was accepted as immunity or old infection and vice versa positive for IgM was accepted as an acute phase of infection. In a case of two of paired sera were positive for IgG, they were titrated by two-fold. Further ELISA IgG of the first and the second sera against TBEV and CCHFV in the same run were made. The differences in four folds between the positive first and the second sera in the same run were considered as an acute phase infection and further investigated for the presence of IgM antibodies.

In case the second serum was positive for IgM, the sera were investigated further by molecular methods of investigation.

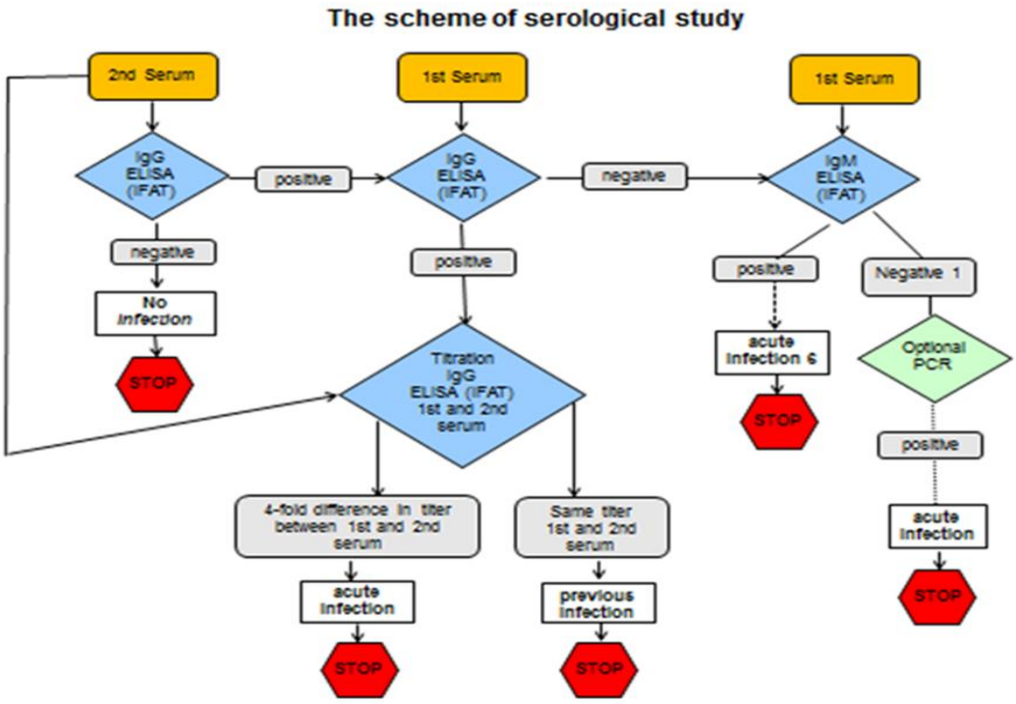


Figure 6.The scheme of sera investigation

4.1.2.9 TBE investigation with ELISA

The paired sera were screened for the presence of antibodies IgG and IgM with ELISA. Antibodies against TBEV were detected by using the ELISA kit (Euroimmun AG, Germany). This test uses the TBEV viral proteins (strain K23) (www.euroimmun.com). Sera samples were diluted 1:100. The human antibodies attached to the viral TBE antigen on the plate. These antibodies were detected by peroxidase-labeled anti-human IgM or IgG. This complex visualized by chromogen substrate solution. The results were considered positive if it exceeded 1.1. The calculation was made according to the manufacturer instructions. The paired samples with OD difference <+0.3 were considered as IgG from the previous infection. But the samples with negative IgG of the first sera were tested for IgM. Due to the flaviviruses could have the cross-reactivity in ELISA detection (145).

4.1.2.10 Immunofluorescence assay (IFA)

Kazakhstan is possibly endemic for other flaviviruses (6). Therefore sera samples tested positive in the ELISA have to be confirmed by other test systems. The immunofluorescence is widely used to detect the cross-reactivity with other flaviviruses (113). Herein, the positive IgG sera samples in ELISA were confirmed by the Flaviren-Mosaik 1(IgG) (Euroimmun, Germany) IIFT according to manufacturerвЂ™s instructions. The IIFT Flaviren Mosaik 1 using the several Bioships coated with TBEV, WNV, JEV and YFV antigens, and the target samples could be tested simultaneously for other flaviviruses. The recommended dilution is 1:10. The positive samples were tested at dilution 1:10, 1:100, 1:200 and 1:300. All slides were investigated in a Microptix immune fluorescence microscope MF300 with 40x ocular.

4.1.2.11 CCHF investigation with ELISA

The all sera were investigated according to the scheme (See shown in annex xx). In a first step, 2nd sera of all participants were screened for IgG with ELISA (Vector-Best, Novosibirsk Russia) (dilution 1:100 as indicated by the kit instruction). Next, for the IgG-positive subset, 2nd and 1st sera then were retested for IgG. Thus, first and second serum samples with OD difference <+0.3 were evaluated as an IgG resulting from a previous infection. Moreover, all other sera were titrated in log2-steps (1:100, 1:200 up to 1:3200). Four-fold titer change was assessed as acute infection. And at the third step, the samples whose 1st sera tested IgG-negative were than investigated by IgM ELISA.

Last, IgM negative and all IgM positive 1st sera these were further investigated by molecular methods (see 4.2.11). CCHFV RT-qPCRs and S and L segments PCRs

The extracted RNA from the sera samples (see 4.8) were screened also for the presence of CCHF RNA (6). But the protocol was adapted 5 Bμl of CCHFV RNA was amplified in 25 Bμl RT-qPCR mixture QuantiTect Virus kit (Qiagen, Germany) with of 0.2 BμM of each primer, forward CCHF S1, reverse CCHFS122 and 0.16 BμM of hybridization CCHF probe with reagents according to the instructions and protocol as it was described before by Atkinson(106).

Table 3. CCHFV Primers for RT-qPCRs (Atkinson et al. 2012)

Primers/Probe	Sequences
S1, Forward primer	TCT CAA AGA AAC ACG TGC C
S2, Reverse primer	CCT TTT TGA ACT CTT CAA ACC
CCHF probe	FAM ACT CAA GGK AAC ACT GTG GCC GTA AG BHQ1

The protocol was optimized on Rotorgen and Quiagen kit (Hilden, Germany).

The amplified products of CCHFV RT-qPCR were run in agarose 1.5% with 100 bp marker and visualized by GelRed. The expected product size was 122 bp. Describe gel here to confirm positive samples.

Samples positive for viral RNA in the RT-PCR were then analyzed by CCHFV specific RT-PCR with primers targeting the S segment forward primer CCHF RWCF) and reverse primer CCHF RWCR(130) and L segments

Table 4. CCHFV primers for conventional S and L segments PCR

Segment	Forward	Reverse
S segment	RWCF CAAGGGGTACCAAGAAAATGAAGAAGGC	RWCR GCCACAGGGATTGTTCCAAAGCAGAC
L segment	F2576 5’-gggAAAATAAggACAgACCA	R3371 5’-TCYgTTAAgCATTCATTRCT

The amplified products were run in agarose 1.5% gel and visualized by Gel Red® under illumination of ultraviolet light.

4.1.2.12 Sequencing

Products of conventional PCR were purified with Qiaquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instruction with the ABI Prism Big Dye Terminator V3.1 Cycle Sequencing Kit and "3500xl Genetic Analyzer" machine using the initial primers of RT-PCR amplification. Retrieved sequences were xxxxx Phylogenetic trees were constructed in MEGA 6 with the Maximum Likelihood method based on the Tamura 3-parameter model (131)(132)(133)

4.1.2.13 Data analyzing and statistics

Hypotheses

We wish to investigate whether the prevalence of CCHF and TBE in Almaty Oblast is above the threshold for the designation of the region as endemic and patients with FUO should be routinely tested for the infection. While the prevalence threshold for a region to be designated as endemic is not officially stated, we take five percent as a reasonable criterion. As, the expected prevalence of TBE should not exceed 5%. We suggested that the calculated sample size will be reasonable for TBE investigation also. Thus, a prevalence level below five percent would be considered as non-endemic and anything above as endemic. Our hypotheses can be stated as follows:

H_0: The prevalence of CCHF infection in Almaty Oblast is 5%, or less.

H_1: The prevalence of CCHF infection in Almaty Oblast above 5%.

Power calculation and the sample size

We wish to collect a sample that would allow us to detect a potential prevalence of two percent above this threshold or more. Thus, if the true prevalence in Almaty Oblast was seven percent of more, we want to have enough data to allow us to reject H_0. The power calculation is for one-sided *z*-test with five percent probability of false positive (Type I error(134)(135), i.e. p-value < 0.05 required for rejecting H_0, is

$$\frac{0.07 - 0.05}{\frac{\sigma}{\sqrt{n}}} > 1.645$$

where the standard deviation of CCHF prevalence under H_0 is at most

$$\sigma = \sqrt{\big(0.05(1 - 0.05)\big)} = 0.218.$$

Plugging this into the inequality above and solving for the sample size, we obtain

$$n > \left(1.645 \frac{0.218}{0.02}\right)^2 = 321.5.$$

Thus, the appropriate sample size for testing H_0 is 322 or more.

Methods of statistical analyzing of the CCHF and TBE seroepidemiological study

The proportion of seropositive patients' calculation and the statistical analyzes to evaluate the independent risk factors for seropositivity were made for both infections. Statistical analyses were conducted by using R 3.3.324. Logistic regressions were used to evaluate the independent risk factors for seropositivity. The criterion for statistical significance was $p < 0.05$. Questionnaire responses from CCHFV and TBEV positive patients were compared with those from CCHFV and TBEV infection negative participants.

Methods of statistical analyzing of results of tick's investigation study

The prevalence of TBEV and CCHFV in ticks was calculated as a minimum infection rate (MIR) assuming that one tick in each pool was definite as a positive. Due to the studied arboviruses have a low prevalence not exceed 5% in investigated pilot regions, the best approach to calculate the prevalence is MIR(136). It is explained that confidence interval of the MIR based on Poisson distribution and it is appropriate if the0 infection level is low. Due to the MIR based on Poisson distribution, the results of the MIR are sensitive for the prevalence of infection, it should not be that only one positive individual is in a total sample size or the prevalence of infection is high. In a case of high prevalence or in a case of only positive individual in total sample, the result of MIR calculation will be invalid and biased(137).

During the project study, the MIR was established by the sampling sites and tick species.

5 CHAPTER 5

5.1 Results

5.1.1 Ticks investigation study.

5.1.1.1 The Ticks investigation

In this study we investigated TBEV in ticks from endemic and non-endemic oblasts in Kazakhstan to obtain the information on the prevalence of TBEV in vectors and circulating subtypes.

Overall, 2341 ticks or 501 pools of ticks were investigated from six regions of Almaty oblast and Kyzylorda. In general, collected ticks were determined as five genera *Ixodes, Dermacentor, Haemophisalis, Ripecephalus* and *Hyaloma*. Thoroughly, *Ixodes persulcatus* (1191 imagoes and two nymphs), *Dermacentor marginatus* (55 imagoes), *Haemophisalis punctata* (470 imagoes), *Dermacentor reticulatus* (14 imagoes), *Dermacentor niveus* (523 imagoes), *Ripecephalus turanicus* (9 imagoes), *Hyaloma asiaticum* (77 imagoes).

In detail In Almaty oblast 1737 ticks were collected or 365 pools. In Tekeli district were collected *I. persulcatus* 610 ticks or 123 pools, *H. punctata* 25 ticks or 7 pools, *D. marginatus* 50 ticks or 12 pools, *D. niveus* 10 ticks or three pools, *D.reticulatus* 14 ticks or three pools. In Yenbekshikazakh district were collected *I. persulcatus* 79 ticks or 16 and one pool of *I. persulcatus* nimphae pools, *H. punctata* 444 ticks or 96 pools. In Talgar district of Almaty oblast were collected *I. persulcatus* 50 ticks or 103 pools *H. punctata* nimphae and one pool respectively (see table 5).

Table 5. The ticks collected in Almaty oblast

Regions	Tick species	Ticks/pools
Tekeli	*Ixodes (I)* persulcatus (Imago)	610 ticks/ 123 pools
	*Haemophisalis (H) punctata (*Imago)	25 ticks/ 7 pools
	Dermacentor (D) marginatus (Imago)	50 ticks/ 12 pools
	D. niveus (Imago)	10 ticks /3 pools
	D. reticulatus (Imago)	14 ticks/ 3 pools
Yenbekshikazakh	*I. persulcatus* (Imago)	78 ticks /16 pools
	I. persulcatus (Nimphae)	1 nimphae/ 1 pool
	H. punctata (Imago)	444 ticks /96 pools
Talgar	*I. persulcatus* (Imago)	504 ticks /103 pools
	H. punctata	1 nimphae / 1pool

In Kyzylorda oblast ticks were collected in three districts Zhanakorgan district, Shiely district and Syrdariya districts at 18 sites, respectively. 604 ticks or 136 pools were collected in Kyzylorda oblast. In Zhanakorgan district were collected *D. niveus* 116 ticks or 25 pools, *H. asiaticum* 77 ticks or 17 pools, *R. turanicus* were collected 6 ticks or two pools. In Shiely district were collected *D.niveus* 119 ticks or 43 pools, *R. turanicus* three ticks and three pools as the ticks were different gender or site of collection. In Syrdariya district were collected *D. marginatus* five ticks or three pools and *D. niveus* 198 ticks or 43 pools (see table 6).

Table 6. Ticks species collected in Kyzylorda oblast

Regions	Tick species	Ticks/pools
Zhanakorgan	*D. niveus (Imago)*	116 ticks /25 pools
	Hyalomma asiaticum (Imago)	77 ticks/ 17 pools
	Ripicephalus (R) turanicus	6 ticks/ 2 pools
Shiely	*D. niveus (Imago)*	119 ticks / 43 pools
	R. turanicus (Imago)	3 ticks/3 pools (different gender and site of collection)
Syrdariya	*D. marginatus (Imago)*	5 ticks/3pools
	D. niveus (Imago)	198 ticks/ 43 pools

The 18 GPS coordinates of the sites of tick collection locations are shown in a map in figure 5. *I.persulcatus* was only found in Almaty oblast in Talgar region next to the mountains. H. punctata were mostly collected in region with meadows in Yenbekshikazakh region. whereas *D. niveus* and *H. asiaticum* tick were only detected in Kyzylorda oblast. D. *niveus* were in all three regions of Kyzylorda oblast but not far from the water source the river Syrdariya, whereas H.asiaticum was collected only in Zhanakorgan far from the source of water (See the pilot regions in Introduction). Hence, *I. persulcatus* was prevailed in Almaty oblast and *D. niveus* and *H. asiaticum* were prevailed in Kyzylorda oblast

5.1.1.2 Investigation of TBEV in ticks

TBEV RNA only was found in ticks collected in Almaty oblast while in Kyzylorda oblast no positive tick pools were found. In detail 51 positive pools (14%) were detected in Almaty oblast. 22 positive pools (6.%) for TBEV RNA positive pools were detected in Talgar region, 22 positive pools (6%) were detected in Tekeli region and only seven (2%) were detected in Yenbekshikazakh region. The Minimum Infection Rate (MIR) of ticks from Almaty oblast (Tekeli, Yenbekshikazakh, Talgar regions) was calculated. The MIR displayed in geographically appearance of TBEV are Tekeli (3.1%), Yenbekshikazakh (1.3%) and Talgar (4.4%) respectivelly. Moreover the MIR divided in different ticks genus are *I. persulcatus* (15.4%), *H. punctata*(0.2%), and *D. marginatus* (2%). Results are shown in a table (see table 7).

Table 7. MIR% by tick species and regions in Almaty oblast, Kazakhstan

Oblast	*Region*	*Number of ticks/pool size*	*Tick species/ number of pools*	*Number of positive(pools in real time PCR by gender*	*MIR % by tick species*	*MIR% by regions*
Almaty oblast	Talgar	505/104	*I. persulcatus* 504/103	♂ 7/49		4.4%
				♀ 15/53	4.4%	
				Nymphs 0/1		
			H. punctata 1/1	♂ 0/0		
				♀ 0/1	0%	
	Tekeli	709/148	*D. marginatus* 50/12	♂ 0/3	2%	3.1 %
				♀ 1/9		
			D. reticulatus 14/3	♂ 0/1		
				♀ 0/2	0%	
			I. persulcatus 610/123	♂ 4/54		
				♀ 17/69	3.4%	
			H. punctata 25/7	♂ 0/4		
				♀ 0/3	0%	
			D. niveus 10/3	♂ 0/3	0%	
	Yenbekshikazakh	523/113	*I. persulcatus* 79/17	♂ 3/7		1.3%
				♀ 3/9	7.6%	
				Nymphs 0/1		
			H. punctata 444/96	♂ 1/43		
				♀ 0/53	0.2%	

In summary 51 TBEV positive samples were further investigated by TBEV specific conventional RT PCR with primers targeting the complete E gene and all positive samples were subsequently sequenced. In detail, in Talgar region 22 pools were

positive for the TBEV RNA presence. But only 14 were positive in E target conventional PCR with primers forward 947 A +947 B and reverse 2579 (primer details see in table 8). In Tekeli region, the 22 positive pools for the presence of TBEV RNA were positive in qRT-PCR and only 19 in target E gene conventional PCR with primers forward 947 A+947 B and reverse 2579 only 19 pools were positive (see table 8).

In comparison to Tekeli and Talgar regions, in Yenbekshikazah seven positive pools the presence of TBEV RNA was detected in real time RT-PCR. But only 5 positive pools were positive for target E gene with primers forward 885 and reverse 2579. However, one pool was also positive with primer combination forward 885 and reverse 2605. (see table 9)

All amplificates of the E.gene had a size of 1687 nt.

Table 8. The positive pools from Talgar and Tekeli regions for target E gene PCR

Region	Tick species	The gender of positive ticks	Number of positive pools/ CT	Target E gene PCR Primers Forward 947 A+947 B Reverse 2579	Sequenced
Talgar	I.persulcatus	♀15 ♂7	22 /CT bellow 39	14	11
Tekeli	I.persulcatus D.marginatus	♀17 ♂4 ♀ 2	22/CT bellow 39	19	15

Table 9. The positive pools from Yenbekshikazakh region for target E gene RT-PCR

Region	Tick species	The gender of positive ticks	Number of positive pools	Target E gene PCR 885 forward and 2579 reverse 885 forward and 2606 reverse	Sequenced
Yenbekshikazakh	*I. persulcatus*	♀3 ♂3	7	5	4
	H. punctata	♀1			

Hence during the sequencing of E gene the 1488 nt open reading frame, 11 sequences of E gene from Talgar region, 15 sequences from Tekeli region (see table 8) and four sequences from Yenbekshikazakh were detected (see table 10).

Table 10. The sequences of E gene of 30 samples and GenBank accession number

No	Year	Gender	Host	Region	GenBank accession number
17	2015	♀	*I. persulcatus*	Talgar	MK284381
21	2015	♂	*I. persulcatus*	Talgar	MK284382
25	2015	♀	*I. persulcatus*	Talgar	MK284383
44	2015	♂	*I. persulcatus*	Talgar	MK284384
45	2015	♀	*I. persulcatus*	Talgar	MK284385
49	2015	♀	*I. persulcatus*	Talgar	MK284386
50	2015	♀	*I. persulcatus*	Talgar	MK284387
54	2015	♂	*I. persulcatus*	Talgar	MK284388
64	2015	♀	*I. persulcatus*	Talgar	MK284389
66	2015	♂	*I. persulcatus*	Talgar	MK284390
68	2015	♂	*I. persulcatus*	Talgar	MK284391
5	2015	♀	*I. persulcatus*	Tekeli	MK284392
28	2015	♀	*I. persulcatus*	Tekeli	MK284393
33	2015	♀	*I. persulcatus*	Tekeli	MK284394
53	2015	♀	*I persulcatus*	Tekeli	MK284395
58	2015	♀	*I. persulcatus*	Tekeli	MK284396

61	2015	♀	*I. persulcatus*	Tekeli	MK284397
99	2015	♀	*I. persulcatus*	Tekeli	MK284398
101	2015	♀	*I. persulcatus*	Tekeli	MK284399
105	2015	♂	*I. persulcatus*	Tekeli	MK284400
111	2015	♀	*I. persulcatus*	Tekeli	MK284401
113	2015	♀	*I. persulcatus*	Tekeli	MK284402
118	2015	♀	*I. persulcatus*	Tekeli	MK284403
120	2015	♂	*I. persulcatus*	Tekeli	MK284404
133	2015	♀	*I. persulcatus*	Tekeli	MK284405
137	2015	♀	*I. persulcatus*	Tekeli	MK284406
75	2015	♀	*I. persulcatus*	Yenbekshikazakh	MK284407
77	2015	♂	*I. persulcatus*	Yenbekshikazakh	MK284408
109	2015	♀	*H. punctata*	Yenbekshikazakh	MK284409
112	2015	♂	*I. persulcatus*	Yenbekshikazakh	MK284410

30 sequences classified our sequences as Siberian subtype of TBEV on nt and aa level. Strain Zausaev was used as a reference strain (see table 10). The Talgar and Yenbekshikazakh sequences are belong to Baltic lineage of Siberian subtype, whereas Tekeli sequences belong to Vasilchenko lineage of Siberian subtype(18). In detail, the sequencing of the 1488nt E-gene open-reading frame leads to 30 E-gene sequences. 11 sequences from Talgar region, four sequences from Yenbekshikazakh region and 15 sequences from Tekeli region of Almaty oblast (see table 11).

Table 11. Amino acid substitutions in the E-protein with the reference strain Zausaev

Strain/aa-pos.	33	41	52	83	88	93	128	150	228	233	277	279	310	313	331	342	384	387	395	430	431	435	460	475	482	487	490	494
Zausaev	I	M	N	A	S	K	T	Y	K	Q	D	T	T	A	T	V	Y	E	K	L	A	K	L	T	L	V	M	V
Konst-14									R												T							
MucAr M14/10																					T							
YuK 4/13																					T							
Kaz-Baidar-S		I										A					H	D	R		T							
Alma-Arasan												A						D			T							
Sib-XJ-X5												A	A	T						F	T	R						
Tal17/15					G	R	I					A		T							T					I		
Tal21/15					G	R	I					A		T							T					I		
Tal25/15					G	R	I					A		T							T					I		
Tal49/15					G	R	I					A		T							T					I		
Tal50/15					G	R	I					A		T							T					I		
Tal68/15					G	R	I					A		T							T					I		
Tal44/15					G	R	I					A		T	A						T					I		
Tal45/15					G	R	I					A		T	A						T	R				I		
Tal54/15					G	R	I				E	A		T							T					I		
Tal64/15	V			V	G	R	I					A		T							T					I		
Tal66/15			T		G	R	I					A		T	A						T					I		
Yen75/15					G	R	I					A		T							T					I		
Yen77/15					G	R	I					A		T							T					I		
Yen109/15					G	R	I					A		T							T	R						
Yen112/15					G	R	I			H		A		T							T		M			I		
Tek05/15												A								F	T	R						
Tek28/15												A								F	T	R						
Tek105/15												A								F	T	R						
Tek58/15												A								F	T	R						
Tek111/15												A								F	T	R						
Tek113/15												A								F	T	R						
Tek118/15												A								F	T	R						
Tek61/15												A							R	F	T	R						
Tek33/15												A							R	F	T	R						
Tek53/15								H				A								F	T	R						
Tek99/15												A								F	T	R		S	R		T	A
Tek101/15												A								F	T	R		S	R		T	
Tek120/15												A							R	F	T	R			R		T	
Tek133/15												A							R	F	T	R					T	
Tek137/15												A				A				F	T	R						

Comparison of the Talgar E-gene sequences with GenBank entries (Stephen F et al, 1997) revealed highest similarities on the nucleotide level (94-95 %, 81-83 nt difference) to the sequence of the strain Buzuuchuk (KJ626343) and on the amino acid (aa) level with the strain MucAr M14/10 (AFU65175) (99 %, 6 aa difference). Comparison of the Yenbekshikazakh E-gene sequences with GenBank entries revealed highest similarities on the nucleotide level (94 %, 84nt difference) to the sequence of the strain Buzuuchuk (KJ626343) and on the amino acid (aa) level with the strain MucAr M14/10 (AFU65175) (99 %, 6 aa difference). Phylogenetic analyses show that both, strains from Talgar and from Yenbekshikazakh are clustering in the Baltic lineage of the Siberian Subtype (see figure 7a). Comparison of the Tekeli E-gene sequences with GenBank entries revealed highest similarities on both, the nucleotide level (99%, 15-21 nt difference) and the amino acid (aa) level (99%, 1-2 aa), to the sequence of Sib-XJ-X5, clustering in the Siberian Subtype (Vasilchenko lineage) (see figure 7a). Phylogenetic analyses of the

496 aa show that all three strains are clustering together in one clade with the strain Sib-XJ-X5 (see figure 7b).

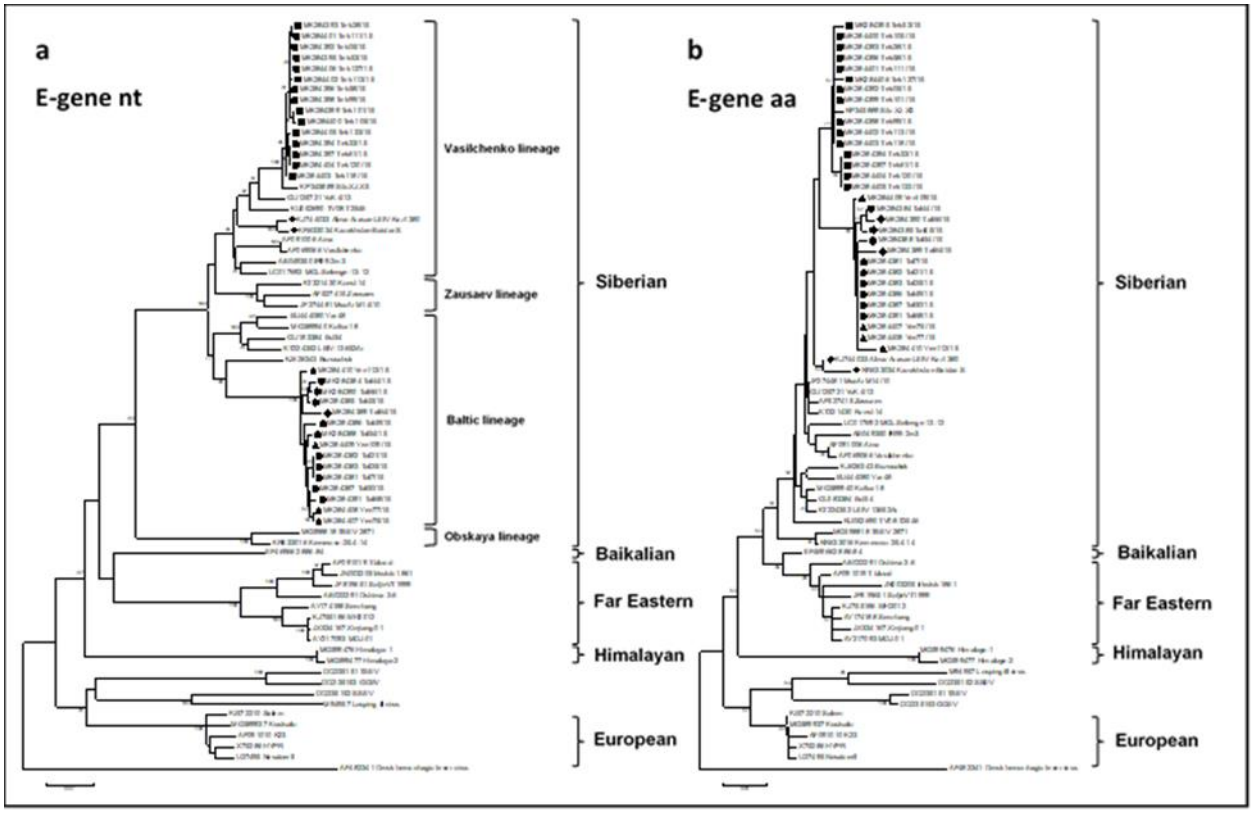


Figure 7. Phylogenic analysis of E-gene of TBEV. Phylogenetic tree illustrating the evolutionary relationships of nucleotide (nt) and amino acid (aa) taxa. The trees were constructed with the MEGA7 software package using the Neighbor-Joining method (Saitou et al.1987, Kumar et al. 2016). The percentage of replicate trees are shown next to the branches, bootstrap test was made in 10.000 replicates (Felsenstein et al. 1085). Omsk hemorrhagic fever virus (AF482341) applied as an outgroup. The newly acquired Kazakh sequences are marked with squares (Tekeli), circles (Talgar) and triangles (Yenbekshikazakh). The former published TBEV sequences from Kazakhstan are marked with diamonds. a) Phylogenetic tree of the open-reading from of the E-gene using 70 nucleotide sequences with the length of 1488nt

5.1.1.3 Investigation of CCHFV in ticks.

All RNAs from ticks were also investigated for the presence of CCHFV RNA by real-time RT-PCR. No positive tick pools the presence of CCHFV RNA were found. All positive controls and negative controls worked.

5.1.2 Seroepidemiological investigation of TBE and CCHF in patients with FUO

5.1.2.1 Enrolled patients

In total, 950 patients were enrolled from 13 hospitals in the two oblasts Almaty and Kyzylorda into the study. However, for 148 patients either only one serum, no

questionnaire or no consent form was available. Therefore only 802 patients with a fever of unknown origin were meeting the criteria, to have paired serum pairs as well as the questionnaire, and were therefore used for the study.

5.1.2.2 TBEV serological investigation by TBEV IgM and IgG ELISAs

According to TBE investigation, it was revealed that the total seroprevalence of TBE infection in Almaty oblast and Kyzylorda oblast was 16 out of 802 (1.9%) and two out 802 (0.25 %) respectively. In the screening for IgM for the acute TBE infection six samples (0.74%) out 802 and two samples (0.25%) out 802 were detected in Almaty and Kyzylorda oblast respectively. In terms of IgG, ten samples (1.24%) were revealed in Almaty oblast and no IgG positive samples in Kyzylorda oblast.

5.1.2.3 Confirmation of results by the Immunofluorescence (IFAT)

According to the immunofluorescence confirmation often TBEV IgG positive samples was indicated that all TBE IgG positive samples are TBEV as in the dilution row only a reaction with TBEV antigen stayed. The light was evaluated between weak and strong with a dilution 1: 10.The positive control for TBEV IgG and for positive control anti-Japanese Encephalitis virus were positive (see table 12).

5.1.2.4 Statistical analysis of seroepidemiological TBEV investigation

The evaluation of clinical symptoms was revealed that patients had symptoms as headache fever (n= 18, 100%), headache (n=17, 94.4%), weakness (n=10, 56%), meningism (n= 5, 28%), exanthema (n=2, 11.1%), diarrhea (n=1, 5.5%). It was also revealed that only two patients out eight with acute TBEV had meningeal symptoms: These were not treated as TBE patients or diagnosed at the hospital according to clinical signs with this infection. All patients with acute TBEV infection had ever higher than 37.5 and lasting more than four days. With regards to the risk factors, we have found that ticks bite in history and living surroundings as occasional bush agricultural fields increase the TBE seropositivity ($p<0.05$). However, the history of contact with any animals and birds, the area of living urban or rural do not affect the seropositivity. The analysis of socio-demographic aspects of the TBEV seropositive proportion of patients shows gender equality among seropositive patients. The mean age of seropositive patients was 32 years old. The analysis of seropositive patients by their occupation revealed that the unemployed were (n=8, 44.4%), students were (n=4, 22%) and skilled laborers were only (n=3, 16.6%)

Table 12. Confirmation of TBEV ELISA results by flavivirus IFAT

Sample number/age/ Gender (	Titers in ELISA IgG (Euroimmun, Germany)	IFA (Euroimmun, Germany)
Almaty 55, 20, f	1:101	1st TBEV 1:300
		2nd TBEV 1:300
Almaty 45, 27, m	1:101	2nd TBEV 1:300
		1st TBEV 1:300
Almaty 10, 24, m	1:101	2nd TBEV 1:10
		1st TBEV 1:10
Taldykorgan,30, 45, m	1:101	1st TBEV 1:300
		2nd TBEV 1:300
Tekeli 13, 64, m	1:101	1st TBEV 1:100
		2nd TBEV 1:100
Tekeli 15, 60, f	1:101	1st TBEV 1:300
		2nd TBEV 1:300
Tekeli 31, 25, m	1:101	2nd TBEV 1:100
		1st TBEV 1:100
Tekeli 38, 19, f	1:101	2nd TBEV 1:200
		1st TBEV 1:200
Tekeli 40, 18, f	1:101	2nd TBEV 1:100
		1st TBEV 1:100
Tekeli 64, 51,f	1:101	2nd TBEV 1:100
		1st TBEV 1:100

5.1.2.5 CCHF ELISA results

The paired sera of 802 samples were screened for the presence of IgG against CCHFV antibodies.

In summary, 378 patients (47.1%) originated from Almaty oblast (not endemic for CCHF oblast) and 424 patients (52.9%) from Kyzylorda oblast (endemic for CCHF oblast). The first screening of second sera revealed 50 samples positive for IgG against CCHF in Almaty oblast and 70 samples in Kyzylorda oblast. In detail, during the first screening into the account, positive and all equivocal samples were taken into account with OD >1 as positive and 0.8-1 equivocal. In a second step for all samples reacting win the CCHFV IgG ELISA a simultaneous screening for the presence of IgG in the first and second sera was performed. The second step revealed that only 62 out 70 (88.5%) samples from Kyzylorda were positive for IgG in both sera and only 44 out of 50 (88%) samples originating from patients from Almaty oblast were positive for CCHFV IgG in both sera. Hence, during the second screening, almost 12 equivocal samples from

Kyzylorda oblast and six equivocal samples from Almaty oblast were negative in the first and second sera. These samples were skipped from further investigation. However, three samples from Kyzylorda oblast were CCHFV IgG negative in first sera and positive in second serum. The following titration of CCHFV IgG-seropositive samples revealed any differences in both samples of 58 samples from Kyzylorda oblast and 44 samples from Almaty oblast. However, in four samples from Kyzylorda oblast, a four-fold difference in TBEV IgG titers was revealed. These samples were further tested for the presence of IgM. IgM antibodies were detected in the second sera of three samples (Kyzylorda 43, Kyzylorda 121, Shiely 26) and one sample (Syrdariya 6) was positive for IgM in both sera (see table 13).

Table 13. The results of sera investigation for the presence IgG and IgM antibodies against CCHFV by ELISA

Oblast	Region	The first screening of second sera for IgG	The second sreening of first and second sera/	Titration Four fold differences	IgM First serum	IgM Second serum
Kyzylorda	Kyzylorda	28	25	2	0	2
	Zhanakorgan	13	13	0	0	0
	Shiely	15	15	1	0	1
	Syrdariya	14	9	1	1	1
Almaty	Almaty	28	28	0	0	0
	Kaskelen	2	0	0	0	0
	Usharal	4	4	0	0	0
	Taldykorgan	7	7	0	0	0
	Kabanbay	1	1	0	0	0
	Tekeli	6	4	0	0	0

Hence, only in three samples (two samples from Kyzylorda and one sample from Shiely) the IgG were equivocal in the second sera but negative in the first, were positive for IgM antibodies against CCHFV in the second sera. These samples were not included in the group of IgG positive samples. Moreover, in the first sera of these samples, CCHFV RNA was detected. Hence all positive serum samples with a four-fold difference in a titration of CCHFV IgG or equivocal in the second for CCHFV IgG antibodies and negative in the first serum for IgG antibodies were screened for CCHFV IgM according to the scheme sera investigation (See 4.2.8). According to this scheme,

one more positive sample for IgM antibodies against CCHFV was detected in Syrdariya. Therefore, from all serum samples included in the screening for acute infections using IgM ELISA four samples (0.5%) were positive for IgM antibodies. Three of these samples (0.4%) from patients with FUO were also positive for CCHFV RNA (See table 14).

Table 14. The results of CCHFV ELISA and RT-PCR in patients with FUO investigated in two oblasts in Kazakhatsn (2014-2015)

Oblast		Almaty	Kyzylorda	Sum
Number of all tested samples		378 (47.1%)	424 (52.9%)	802
Number of seronegative samples		334/802 (41.6%)	362/802 (45.1%)	696 (86.8%)
Number of IgG sepositive samples		44/802 (5.5%)	58/802 (7.2%)	102/802 (12.7%)
Number of IgM positive samples	1st serum	0/802 (0%)	1/802 (0.1%)	4 (0.5 %)
	2nd serum	0/802 (0%)	3/802 (0.4%)	
Prevalence of seropositive samples by oblasts		44/378 (11.6%)	62/424 (14.6%)	106/802(13.2%)
Number of RT-qPCR positive samples (%)		0/802 (0%)	3/802 (0.4%)	3/802 (0.4%)

5.1.2.6 Acute infection of CCHF

During the screening of all positive sera for IgM antibodies against the CCHFV, it was revealed that four sera samples were positive for IgM antibodies against CCHFV. The second sera of three samples were positive for IgM (Kyzylorda 43, Kyzylorda 121 and Shiely 26). These three samples are unique as CCHFV RNA was detected in the first sera. Also, one serum was positive for IgM (Syrdariya 6) but the first serum. Among these four positive samples, three patients (Kyzylorda 121, Shyeli 26 and Syrdariya 6) had the subclinical manifestation of CCHF.

Only one patient positive for IgM against CCHF (Kyzylorda 43) developed the CCHF with specific clinical manifestation as a hemorrhagic syndrome. In detail, the patient was a woman born in 1993, developing the disease five days after a tick bite. The tick bite was on the left shoulder on 20.07.2015, and on the same day, the tick was removed by the patient. The disease onset was on 25.07.2015 with high temperature, heavy, and muscle pain. The patient took Acetaminophen (Paracetamol). Due to the health deterioration, she went to the village hospital, and she was sent to the regional

infectious disease hospital with clinical diagnosis Rickettsiosis. Only at the regional infection disease hospital, the patient was hospitalized to the intensive care department with diagnosis "Crimean-Congo hemorrhagic fever" as during the examination the signs of the hemorrhagic syndrome were revealed. The appearance of the hemorrhagic syndrome was presented as hematomas and nasal bleeding. On the day of admission in the blood analysis was revealed thrombocytopenia. The blood analysis on the day of admission 27.07.2015, (19-35 PM) - Thrombocytes - 230- 10*9 / L (Norm- 180-320), Leukocytes -4.4 - 109 / l, ESR (Erythrocytes sedimentation rate)-13 mm / h, RBC (red blood cells) 4.5, Hemoglobin-120 g / l. The level of thrombocytes decreased and reached on the fifth day of the disease 30.07.2015 Blood - thrombocyte-38- 10*9 / l, Leukocytes -1.9 – 10*9 / l, ESR-26mm / h, RBC-3.9, Hemoglobin -118 g /l. The level of liver enzymes was within the norm. The diagnosis was proved with CCHFV-specific ELISA and RT-PCR.

In three samples CCHF RNA was revealed. The S and L segments were characterized with sequence analyzing. The phylogenic analysis was performed (4.3.1.2). The studied sequences were aligned with reference sequences of S (180 nt) and L (220 nt) segments. According to S segments the CCHFV groups into seven clades according to the geographical distribution (1.West –Africa, 2. Central Africa, 3.South Africa, and West-Africa, 4.Middle East and Asia, 4.Asia 1, 5.Asia 2, 6.Europe Clade, 7.Greece). According to L segment the CCHFV topology the same as with S segment(138). Hence the analysis of the partial S and L segments of three samples (Kyzylorda 43 (Kyz 43), Kyzylorda 121 (Kyz 121), Shiely 26 (Sh 26) were conducted. The gene bank accession of S segments Kyz43 MG974100, Kyz121 MG974101, Sh26 MG974102 and of L segments Sh26 MG974103, Kyz43 MG974104. In the analysis of the partial S Segment, all three detected CCHFV RNAs from Kazakhstan clustered in subgroup Asia 2 (see figure 8). Analysis of partial L segments of the sample Kyzylorda 43 revealed a clustering in subgroup Asia 2 with strains from Tadjikistan (AY20893, KX013444) and a strain from 1971 from Kazakhstan (KX01354453). However, the partial L segment from sample Shiely 26 clustered in subgroup Asia 1 with strains from Iraq, Afghanistan, and India. Therefore, for the sample Shiely 26, recombination between the two Asian subtypes of CCHFV is present (see figures 9).

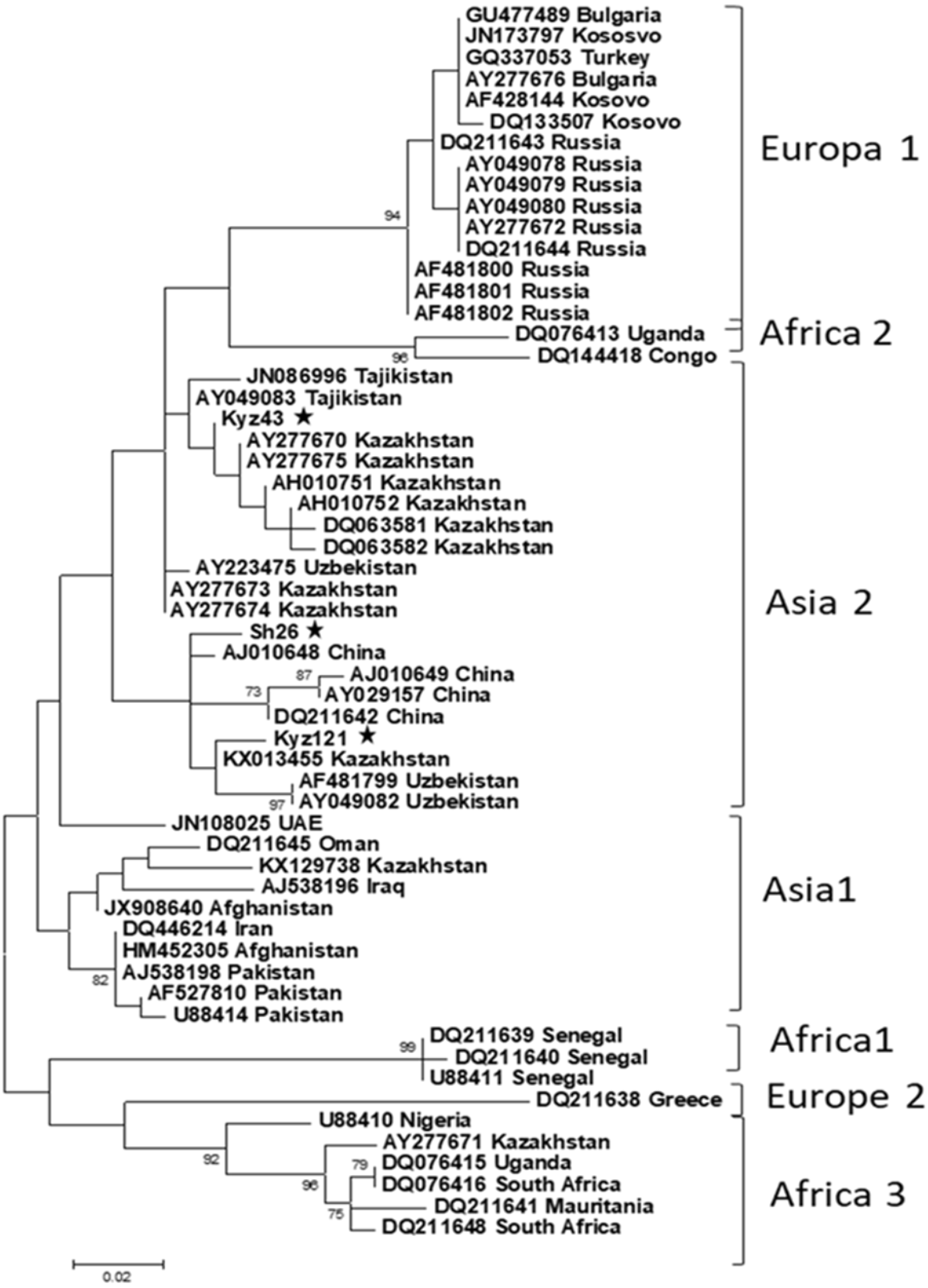


Figure 8. Phylogenic analysis of the partial Small (S) gene fragment of CCHFV.

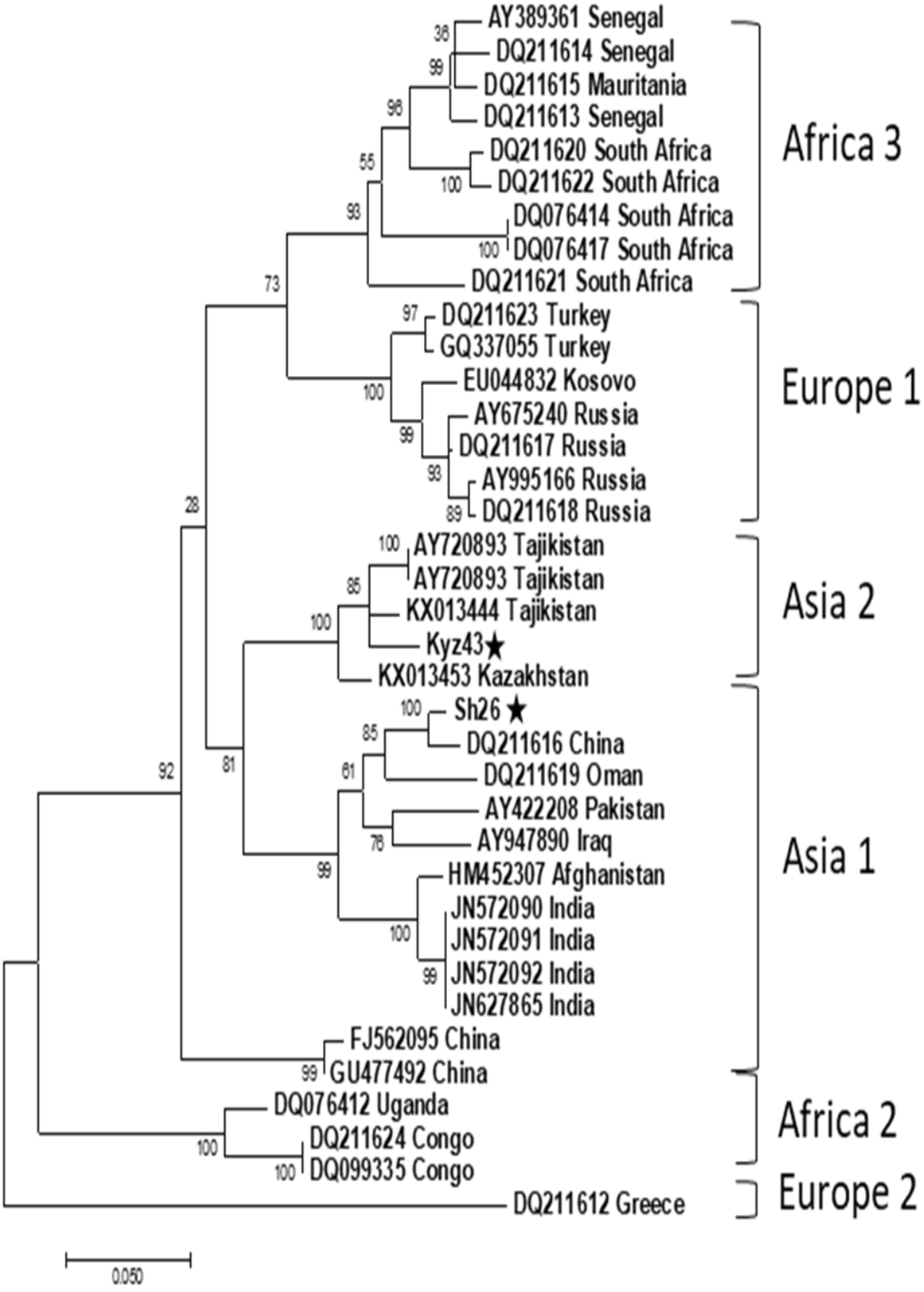


Figure 9. Phylogenic analysis of the partial Large (L) gene fragment of CCHFV

5.1.2.7 Statistical analysis of seroepidemiological CCHF investigation

Despite the clinical appearance of seropositive patients, the questionnaires showed that patients had only not specific and mild symptoms such as headache (n= 99, 93.4%), fever (n= 104, 98.1 %), weakness (n= 81, 76.4 %), exanthema (n= 33, 31.1%), and diarrhea (n= 13, 12.3%).

The proportion of seropositive patients' calculation and the statistical analyses to evaluate the independent risk factors for seropositivity were made for both infections. Statistical analyses were conducted using R 3.3.324. Univariate logistic regressions, reported in table 1, did not identify a statistically significant difference in the prevalence of seropositivity between males and females. Seropositivity is also unrelated to age. Urban areas tend to have lower seropositivity than rural areas; however, this result is only statistically significant at the 10% level.

Concerning the risk factors, we have found that animal husbandry increases the prevalence of seropositivity ($p < 0.05$). However, history of tick bites or presence of birds does not affect seropositivity. It has obtained similar results using univariate OLS regressions with robust standard errors. Finally, breaking down the animal husbandry, we have found that, with the exception of pigs, higher frequency of contact with all other animals for which we have collected data (sheep, goats, cattle, horses, cats, and poultry) leads to an increased prevalence of seropositivity, however, the effects are only statistically significant for cattle and horses. The univariate logistic regression identified that the seropositivity significantly unrelated to the patient's occupation as students, farmers, housekeeper, driver, unskilled labor, administrative workers, workers of a medical facility, people in business, farm workers who work with plants. However, the univariate logistic regression detected statistically significant relation only between seropositivity and farmers who work with animals $p<0.05$ (see table 15)

Table 15. Characteristics of CCHFV seropositivity in patients with FUO in Kazakhstan (2014 – 2015)*

Variables	Seronegative IgG (n = 696)	Seropositive (n = 106)	Univariate logistic regressions explaining seropositivity	
			Logistic (change in OR)	OLS (change in probability)
Gender:				
Male	427	64	-0.071 (p> 0.05)	-0.008 (p> 0.05)
Female	261	42		
NA	8	0		
Age:				
Mean	35	35	0.005 (p> 0.05)	0.001 (p> 0.05)
Standard deviation	16	15		
History of tick bites:				
Yes	159	20	-0.253 (p >0.05)	-0.028 (p >0.05)
No	531	86		
NA	6	0		
Animal husbandry:				
Yes	290	48	0.508 (p <0.05)	0.059 (p <0.05)
No	399	58		
NA	7	0		
Birds or nests at home:				
Yes	97	19	0.061 (p >0.05)	0.008 (p >0.05)
No	399	97		
NA/does not know	409	0		
House location:				
Rural area	328	60	-0.364 (p> 0.05)	-0.042 (p> 0.05)
Urban area	362	46		
NA	6	0		
Occupation				
Student/Pupil	97	17	0.446 p>0.05	0.048 p>0.05
Farmeworker				
Plants	11	2	0.495 (p>0.05)	0.186 (p<0.05)
Animals	10	4	1.283 (p<0.05)	0.186 (p<0.05)
Forestry	8	1	0.063 (p>0.05)	0.006 (p>0.05)
Housekeeper	27	6	0.665 (p>0.05)	0.079 (p>0.05)

Unskilled Labourer	40	8	0.579 (p>0.05)	0.066 (p>0.05)
Driver	20	5	0.813 (p>0.05)	0.100 (p>0.05)
Administraion/Academic professional	55	8	0.264 (p>0.05)	0.027 (p>0.5)
Business man/woman	17	2	0.005 (p>0.05)	0.0009 p>0.05
Nurse/Physician/Pharmacist	19	1	-1.089 p>0.05	-0.085 p>0.05
Other profession	124	14	0.013 p>0.05	0.001 p>0.05
Unemployed	137	24	0.443 p>0.05	0.048 p>0.05

6 CHAPTER 6

6.1 Discussion

6.1.1 Investigation of TBEV in ticks

One of the primary aims of this thesis was the investigation of TBEV and CCHFV in vectors.

The first aim was to investigate TBEV in ticks; in detail, to estimate the prevalence in ticks, a circulating subtype and to describe the TBEV vectors.

Hence, in Kyzylorda, four species of ticks were detected *D. niveus, D. marginatus*, *R. turanicus* and *H. asiaticum*. Our findings are consistent with previous studies on ticks performed in Kazakhstan. In these studies, seven species of ticks were described in Kyzylorda as *D. niveus, D. marginatus, R. turanicus, H. asiaticum, H. punctata, Hyalomma suspense, Rhicephalus pumilio* (36). Our study demonstrated that tick species *D. niveus* (111 pools) was dominant among other species *D. marginatus* (3 pools♀), *H. asiaticum* (13 pools♂ and 4 pools ♀), *R. turanicus* (3pools♂, 4 pools ♀). Following our data, tick species *D. niveus* (111 pools) is dominant in this region. It could be explained that the prevalence of tick species is affected by the peak of activity, time of tick sampling and geographical distribution of ticks *D. niveus*. *D. niveus* inhabit mostly the flood plains as they need the optimal level of humidity for the living and oviposit and altitude not more 1500 meters above the Sea (139). The structure of Haller' organ could explain the geographical inhabiting of flood plains by *D. niveus*. In contrast to *H. asiaticum*, Haller's organ of *D. niveus* has not mature glands that could provide the constant level of moisture continent (140). Also, the tick sampling was conducted at the pick activity of *D. niveus*. The pick of activity of *D. niveus* in Kazakhstan is in April, March, and May. During these months, the temperature on average is mild. Moreover, the weather is still humid this time of year (141). The tick sampling was conducted in Kyzylorda oblast in an area of Syrdariya river flood plain that has favorable conditions for *D. niveus*. In contrast to *D. niveus* the activity of *D. marginatus* (5 pools), *R. turanicus* (3 pools) appeared later. Moreover, *D. marginatus* inhabit mostly steppes and semi steppe regions, and inhabiting does not require only the flood plains and can range until 3000 meters above the sea (142). Our findings on *H. asiaticum* (17 pools) reflect the time of tick activity and results of previous published studies conducted in this region. The tick activity of *H. asiaticum* in this area starts later and last till the end of July, as this species more adaptive for dry and hot weather because of the structure of cuticle and Haller’s organ, the glands of Haller’s organs providing good moisturizing

(142). 64 pools from Almaty oblast were investigated and five tick species *I. persulcatus* (131 pools♀, 110 pools♂, 2 pools nymphs), *H. punctata* (110 pools ♀, 47 pools ♂), *D.niveus* (3 pools♂), *D. marginatus* (9 pools ♀, 3 pools♂), *D. reticulatus* (2 pools♀, 1 pool ♂) were found. The observation over the ticks in our study revealed that the tick species *I. persulcatus* (242 pools), *H. punctata* (104 pools) have prevailed in contrast to other tick species collected in Almaty oblast. According to the previous published tick studies , *I. persulcatus* and *H. punctata* are endemic in Almaty region as they prefer humidity and can inhabit the territories with an altitude up to 2000 meters above the sea level (140). Almaty oblast was shown to have a favorable condition for the appearance of the ticks of the species *I. persulcatus* and *H. punctata* (8). The distribution of ticks *D. niveus, D. marginatus, D. reticulatus* in Almaty oblast were also described in previous published studies (141). Hence, our data reflects the recent results on the distribution of tick species in Almaty region.

The collected ticks were investigated for the presence of TBEV RNA. In Kyzylorda all investigated RNA from all tick pools were negative for TBEV. This could be explained by the absence of the main vector of TBEV, the tick species *I. persulcatus* (61) Features of the natural landscapes might influence the dearth of *I. persulcatus* in Kyzylorda oblast. The landscapes such as deserts and steppes with lack of humidity are not favorable for *I.persulcatus*.(61). Despite recently the evidence of TBEV was also found in other tick species such as *H. asiaticum, D. niveus and R. turanicus* in Kazakhstan (61) we did not find TBEV RNA-positive pools of *D. niveus, H. asiaticum and R. turanicus* in Kyzylorda oblast.

In comparison to Kyzylorda Oblast, in Almaty oblast, TBEV RNA was revealed in three tick species *I. persulcatus, D. marginatus, and H. punctata*. Previously published results by Japon demonstrated that ticks *Dermacentor* and *Haemophisalis* could be vectors of TBEV(143)

Moreover, the findings of the study consistent with results of a previously conducted investigation in Kazakhstan; the evidence of TBEV infection in ticks species *D. niveus and D. marginatus* were demonstrated with TBEV antigen ELISA. These studies were engaged in Almaty and North Kazakhstan oblasts of Kazakhstan (144).

Following this, in our study, we can confirm that the endemicity of TBEV in ticks depends on the prevailed tick species *I. persulacatus*

The MIR among positive tick species of *I. persulcatus, D. marginatus*, and *H. punctata* were 15.4%, 2%, 0.22% respectively; thus a higher MIR was detected in the regions of Almaty oblast in *I. persulcatus*. The MIR in Talgar, Tekeli and

Yenbekshikazakh regions of Almaty oblast are 4.35%, 2.82%, and 1.4% respectively. Only limited data on the TBEV MIR in ticks of investigated areas in Almaty oblast is available. Nevertheless, the MIRs in our study reflect the prevalence of TBEV in ticks in endemic regions in bordering countries. The prevalence in ticks ranged between 0.5% and 10.2% in Siberia (145) and 1.6% and 1.3% in Mongolia (146) (54). According to previous studies based on antigen detection with direct hemagglutination reaction conducted in Kazakhstan in 1970 the prevalence of TBEV in ticks reached 30% (63).

In this project study we have used rt-RT-PCR. The MIR was up to 5%. It was explained that diagnostics methods were different. Previously used methods of investigation based on antigen detection with direct hemagglutination reaction or antigen ELISA. These methods have lower specificity than the real-time RT-PCR we used in our study(47) (147). Moreover, it was revealed that it has also higher sensitivity than conventional RT RT-PCR. It could be affected by amplification of shorter fragment (approximately 68 nt) in real time RT-PCR than amplification of longer fragment (approximately 1687 nt) in conventional RT-PCR(145) However, we could not exclude the fact that primers did not match perfectly the sites of amplification. So, sequence and subsequent phylogenetic analyses of 15 samples from Tekeli, eleven samples from Talgar and four samples from Yenbekshikazakh regions classified our isolates as the Siberian subtype of TBEV (figure 6). Comparisons of E gene of Siberian and Far Eastern subtype revealed that the virus strains from Tekeli are located within the Vasilchenko lineage of Siberian subtype and the strains from Talgar and Yenbekshikazakh are located within the Baltic lineage of the Siberian subtype. The comparison of the E gene sequences of our samples from Tekeli revealed highest similarities with the virus strain Almaarasan(60) which also was isolated in Almaty Oblast. Eleven virus sequences from Talgar and four from Yenbekshikazakh are closely related to the strain Buzuchuk and belong to the same lineage (148). Buzuchuk is an area in Kyrgyzstan close to the border of Kazakhstan. The Tekeli samples and Almaarasan from Almaty belong to the western cluster of Siberian subtypes because they are closely related to this cluster whereas the strains from Talgar and Buzuchuk are related to strains from Eastern Siberia. According to the data we got in our study we can hypothesize that strains could be distributed by birds more than 20 bird species are cross-border. These birds species could nest in Kyrgyzstan and fly to Kazakhstan and vice-versa (149) (150). Animals or along manmade routes like motorways or railways from the North to the South as an example the route from Siberia to China or Pakistan through Kazakhstan or vice versa (151). An important role of the strain distribution could

play bird migration. It has already shown in several studies that birds are able to carry the ticks over long distances (57) (152)(153)(154)(155). In Kazakhstan, during the last years due to the bird migration of birds the geographic borders of the bird fauna was changed. Some birds in the 50s of last century were only seen at the borders of the Southern Part of Kazakhstan, but now they became the main bird species over the whole territory (149) that could be a reason of spreading TBEV. Following this, since 2017 the cases of TBEV has been registering in the North part of Kazakhstan. The role of animals is also a significant factor in TBEV distribution. Further ticks parasitize on mammals and could be carried by them by animal transports and transport the TBEV infected ticks into not endemic areas (156).

6.1.2 The investigation of CCHFV in ticks

The second question of this thesis was to look at CCHFV in ticks. Any CCHFV positive pools were found in Almaty and Kyzylorda oblast. The negative pools for the CCHFV RNA presence in Almaty oblast could be explained by not endemic area for CCHFV and absence the main vector of CCHFV ticks *spp. Hyalomma*(61).

6.1.3 Seroepidemiological study of TBEV infections.

One of the major aims of this project study was to know the proportion of TBEV seropopositive patients among the patients who suffer from fever of uknown origin. Clinical presence of TBE could vary and manifestate with unspecific symptoms as fever to fatal neurological disorder (25). Hence the paired sera of 802 patients were screened for the presence of antibodies against TBEV.

The seroprevalence of TBE ranged between endemic Almaty oblast and not endemic Kyzylorda oblast, 1.9 % and 0.25 % respectively. In bordering Mongolia, serological investigations revealed approximately the same percentage of antibodies against TBEV in human samples (Selenge 5.1%, Bulgan 0.9%) (166).

In Almaty oblast, we have found 1.24 % of IgG against TBEV that could be considered as the evidence of recent infection or immunity because of vaccination. The questionnaire did not include a question over a conducted vaccination against TBE. However, I cannot claim that the IgG seropositivity is an outcome of vaccination against TBEV as an only limited part of the population in Kazakhstan is vaccinated against TBEV according to the Kazakh order number 562 of October 1, 2013, on Vaccination against Tick-borne Encephalitis (https://egov.kz/cms/ru/law): preventive immunization is performed to contingent of the population, which includes persons of

any profession whose work is connected with staying in the natural focus of tick-borne encephalitis. Therefore most of the Kazakh population is not vaccinated against TBEV. Moreover according to some studies TBE could manifestate only with fever (26). During our project study we revealed only five patients among 18 seropositive who had meningeal symptoms. All patients suffered from temperature and they were not diagnosed as TBE. This gives the thought that in general most of TBE cases remain unregistered as patients often have only mild or no symptoms.

Also, we revealed 0.75% of patients with IgM antibodies against TBEV in Almaty oblast and 0.47% (two patients) in Kyzylorda oblast. Hence, the results of the serological investigation of TBEV demonstrates that the seroprevalence of in Almaty oblast is higher than in Kyzylorda oblast. The findings of IgM antibodies against TBEV show evidence of acute infection. If in the case of Almaty oblast it could be expected as Almaty oblast is endemic for TBEV(61) (145)(10)., but the findings of acute TBEV infection in Kyzylorda oblast is a casuistry. However, the findings in Kyzylorda oblast of two IgM TBEV positive samples indicate, that TBE should be considered more carefully in Kyzylorda oblast as this is so far not notified as an endemic oblast for TBEV.

More detailed investigation of risk factors of these two cases revealed that both of them have often contact with animals; no one noticed the tick bite or visited for TBEV endemic regions.

Moreover, positive ELISA findings could be due to the cross-reactivity with other flaviviruses (117) (113) and require more deeply investigation and require confirmation by IFA or better neutralization assay. Due to the manufactures for ELISA virions inactivated with formalin are usedas antigens and these virions are directly coated on the plate. Although the manufactures give the high numbers over specificity, the results could be falsely positive because of cross-reactivity among flaviviruses. This is especially often reported in regions endemic for more than two flaviviruses e.g. Powassan, West Nile and Karshi viruses are endemic for Kazakhstan and Turkmenistan; in bordered Uzbekistan is evidence of circulation of Karshi and West Nile were described. These viruses belong to the flavivirus genus and could be also a reason for fever and encephalitis(6) (49)(50). Hence, the cross-reactivity among flaviviruses and low specificity is a challenge for ELISA as previously, in experimental studies was investigated that due to the formalin-inactivated virions coated on the plate, the specificity could be decreased. Formalin-inactivation alter the Protein E, it leads to the formation of covalently cross-linked protein E oligomers and therefore change the specific epitopes and affects the reactivity of antibodies (51)

However, the positive TBE IgM samples in Kyzylorda oblast could be true TBE findings as we confirmed all positive samples with IFA. On the one hand these positive samples of TBE from Kyzylorda could be the evidence of reactivation of semi-desert foci of TBEV, that were found in the 70s during the virological investigations of semi-desert territories of the southern parts of Kazakhstan (145)(187)(188). But on the other hand TBEV carrying ticks could be transported with birds or animal from TBEV endemic region in non-endemic regions (152)(156)

6.1.4 Seroepidemiological investigation of CCHF infections

Clinical manifestation of CCHF varies from unspecific symptoms such as FUO to fatal hemorrhagic fever (73)(157). FUO is a significant public health issue as it is seldom adequately diagnosed and therefore the treatment of patients is insufficient (158)(2)(159)(1). Our study gives serological and molecular evidence that asymptomatic forms of CCHF are high in patients with FUO investigated in Kazakhstan. Kyzylorda is a well-known endemic region for CCHFV (127)(160)(61). However, our results show that asymptomatic CCHFV infections with mild and not specific symptoms also circulate in the non-endemic Almaty oblast. The rate of seropositivity and the symptoms correspond with those previously reported for CCHFV-endemic regions in the Balkans or Africa.(157)(161)(162)(158)(163)(164)(165). These findings are consistent with previous studies, which also found that, in a certain proportion of patients, the clinical manifestation of CCHF in endemic countries can appear without specific symptoms, so only with fever(165). Moreover, in a classification of clinical form of CCHF by Leshinskaya (1970) adopted in Kazakhstan a non-hemorrhagic form of CCHF takes place also. Some previous studies based on an indirect hemagglutination test for antigen detection and compliment fixation test for antibody detection were conducted in Kazakhstan in 1980ies. He it was shown that non-hemorrhagic forms of CCHFV could appear and reach up to 17% prevalence in CCHFV endemic regions (128).

However, in this work four patients were IgM positive and in three of these the viral RNA was detected from patients from Kyzylorda oblast. So, the mild atypical symptoms appeared not only in patients with recent evidence of infection but also in patients with the evidence of acute disease. Importantly, the unexpected IgM-positive samples from Kyzylorda, confirmed with PCR(106), corroborate the conjecture by Christova et al. that CCHF could appear as an asymptomatic infection(158). The big debates upon IgG seropositivity and possible clinical forms of CCHF, as some authors suggest that CCHF could have "a subclinical form" of the disease without any clinical symptoms and we

could find only the evidence of recent infection in a certain proportion of the population(163).

The CCHF ELISA was conducted by “Vector-Best” Kit. The “Vector-Best” manufacture gives around 100% of sensitivity www.vector-best.ru. However, according to some studies the sensitivity of IgM and IgG "Vector-best" is much less, 87.8% and 80.4% respectively (113). However, according to the study, the given sensitivity 100% could match the Kazakh samples as "the Vector-Best" manufacture used the region oriented strains (166).

The positive CCHF samples were not tested by e.g. a confirmatory IFA - in comparison to the serological investigation of TBEV. But this was not needed as CCHFV RNA was detected in positive IgM samples.

The evaluation of independent risk factors and CCHFV seropositivity shows that the rate of CCHFV seropositivity is higher in people with a history of contact with animals and occupations linked to the animal husbandry ($p<0.05$). Previous studies also reported that people could be infected only in occasional contact with livestock. The overlap of CCHFV seroprevalence in animals could lead to the incidence in human (167)(84). It was revealed that mostly all animals with the exception of pigs lead to an increased prevalence of CCHFV seropositivity, however the effects are only statistically significant for cattle and horses. This could be explained that most of the population in the investigated cohort are engaged in animal husbandry. Kazakhstan is an agro-industrial country, and the animal husbandry is a traditional branch of agriculture (www.articlekz.com). It was also revealed that an independent risk factor as a tick bite unrelated to the seropositivity. It could be explained that usually people could not feel a tick bite or could manifest with different skin lesions (168) (see table 15). The risk factors that increased CCHFV seropositivity reflect the risk factors for CCHFV described in previous studies. For example the contact with animals and occupation as a farmer (animal husbandry) significantly increase the CCHFV seropositivity (169)(170). Although previous studies describe that tick bites is a risk factor that significantly increases seropositivity, we were not able to reveal the association of the CCHFV seropositivity with tick bites during this study (169)(171)(172)(84)(119).

6.1.5 Molecular investigation of positive sera for CCHFV

The results of the sequence of S and L segments of two CCHFV-posiitve samples from Kyzylorda show that they clustered within the CCHFV Asia 2 subgroup, whereas the sample Shieli 26 is within the CCHFV Asia 1 group. Previous studies report that

these two subgroups are present in Kazakhstan(173). Although the close relation between the S segment phylogeny and territory is reported and the territory of Kazakhstan does not adjust to any countries with circulation Asia1 subgroup. Our findings support data previously described in Barry Atkinson thesis, 2016, that there is evidence of circulation of different CCHFV subgroups on the same territory (6). Despite in previous papers 12 sequences were described in Kazakhstan and they belong to Asia 1, Asia 2 and Africa 3 subgroups, never the reassortant of the CCHF virus was described previously. This study revealed that the sample Shieli 26 contains a recombinant belonging to Asia 1 subgroup; in detail the S segment sequence analysis of three (Sh26, Kyz43, Kyz121) clustered within Asia 2 group and one sample (Sh26) is reassorted genotype Asia 2 in S segment and Asia 1 in L segment. The recombination is a way of RNA viruses to generate the genetic assortment and refer to some hypothesis it is a way of virus defense to circumvent the immune system(174). The recent study shows that the virus migration even in local distance plays a statistically significant role in virus transmission and virus recombination (175)(176). Therefore, bird migration could play a significant role in the transmission of the virus and explain the presence of Asia 1 subgroup in Kyzylorda oblast (65)(175)(176).

6.2 Limitations of project study

6.2.1. Tick study

Despite the previously conducted training with specialists of the organizations who collected the ticks and delivered them from the remote areas, the ticks that we investigated in some cases were delivered with violation of the delivery regime, especially from the remote areas as Kyzylorda oblast. The violation of the delivery regime affected the results of molecular investigations in case of RNAs investigation, as RNAs are very sensitive and could be destroyed very quickly. Also, in some cases, the critical data as coordinates of tick collection were wrongly marked.

6.2.2. Serological study

Despite the conducted training of medical facilities to collect the blood from the, collecting two pairs of sera and patients support in filling the questionnaires, not all samples met the criteria to be included in the study. The limitation of this study was the collection of data. Due to the lack of continuity among medical organizations and the governing bodies, scientific institutions the collection of blood data were difficult. The

planned time for blood collection was prolonged from 1 year to 2.5 years. Hence after this time, 950 sera samples were delivered for investigation, but only 802 serum pairs met the criteria of inclusion and could be investigated further for TBEV and CCHFV

One of the significant limitations is a cross-sectional study design of this performed seroepidemiological study. A cross-sectional study has its intrinsic susceptibility to bias because of misclassification due to recall biases and due to low response. Hence some possible influences on seropositivity as the living conditions, the possible contact with animals, tick bites were not measured impartially. Moreover, due to the cross-sectional design of the study, there was a limitation of its inherent ability to make the cause-effect relationship (177).

6.2.3.Molecular methods of investigation

Due to the lack of data on previously circulated TBEV subtypes in Kazakhstan, the selection of adequate primers to succeed to get the E-gene for molecular characterization of the TBEV strains was undertaken. The primers and protocols for target E gene RT-PCRs were further developed in this study and updated.

During the molecular investigation of CCHFV, the protocol of the RT-PCR was updated. The previous protocol was suggested by Atkinson et al. 2012 by applying the LightCycler 2.0 and LightCycler 480 machines (Roche Diagnostics Corporation, Indianapolis). During the study, the protocol was optimized on Rotorgen and Qiagen kit (Hilden, Germany). Due to optimization, many false positive results were detected. Troubleshoot was the confirmation of all positive samples by running in agarose 1.5% gel and GelRed.

7 CHAPTER 7

7.1 Conclusion

In summary, I described in detail data regarding TBEV circulation in Almaty oblast in Kazakhstan. For the first time, I gave the information about the MIR of TBEV in ticks in endemic (Almaty) oblast being up to 4.4%. Moreover, for the first time, the subtype of circulating TBEV was determined; in detail, the sequence analyzing revealed that 15 Tekeli samples belong to Vasilchenko lineage, four Yenbekshikazakh samples and 11 samples of Talgar samples belong to the Baltic lineage of Siberian subtype.

Also, for the first time in Kazakhstan was conducted the seroepidemiological study on the investigation of CCHFV and TBEV. The interesting findings were the detection of acute TBE in patients with fever of unknown origin in not endemic for TBE oblast. This data gave a thought to investigate further TBEV circulation in not endemic oblasts and get the full genome sequence of TBEV. Hence, this project study was a basis for further investigations in this field. Currently is another Ph.D proposal discovering the question of circulation and clinical aspects of TBE in not endemic for TBE Akmola oblast; In detail ticks and human samples are investigated from Akmola oblast.

Moreover, for the first time, in Kazakhstan was described that acute CCHF could appear as asymptomatic infection in a certain proportion of the population in the endemic region. Molecular methods of investigation proved this finding. Four patients were IgM positive and in three of these the viral RNA was detected from patients from Kyzylorda oblast. So, the mild atypical symptoms appeared not only in patients with recent evidence of infection but also in patients with the evidence of acute disease. Therefore, these IgM positive patients were unexpected and findings during the study. Also, for the first time, we detected the recent CCHF infection in patients with fever in not endemic Almaty oblast. For the first time, we described the information about the distribution of CCHF and the prevalence of CCHF in Kazakhstan. Also, for the first time, TBE was investigated in endemic and not endemic oblasts (areas). If in a case of endemic Almaty oblast, it was expected the proportion of TBE positive patients up to 2 %, but for Kyzylorda oblast, it was a casuistry. Hence we are for the first time reported about the acute TBE cases (0.25%). The results of this project study show that the distribution of CCHF and TBE could be broader, and be the reason for FUO. FUO should be a significant aware for physicians who face with fever during the tick activity.

7.2 Recommendations

According to the results of study projects the improvement of surveillance system over the CCHF and TBE is recommended. It is recommended to implement the molecular methods of investigation of vectors and in an investigation of TBE and CCHF suspicious cases among humans that were worked out during the project study. It is recommended to investigate other areas with suspicious cases as the distribution of these infections could be broader. Moreover, public health education over the transmission of these infections is also recommended.

References

1. McGregor AC, Moore DA. Infectious causes of fever of unknown origin. Clin Med J R Coll Physicians London. 2015;15(3):285–7.
2. Bodur H, Akinci E, Ascioglu S, Öngürü P, Uyar Y. Subclinical infections with Crimean-Congo hemorrhagic fever virus, Turkey. Emerg Infect Dis. 2012;18(4):640–2.
3. Elyan DS, Moustafa L, Noormal B, Jacobs JS, Aziz MA, Hassan KS, et al. Serological evidence of Flaviviruses infection among acute febrile illness patients in Afghanistan. J Infect Dev Ctries [Internet]. 2014;8. Available from: http://www.jidc.org/index.php/journal/article/view/4183
4. Gould E, Pettersson J, Higgs S, Charrel R, de Lamballerie X. Emerging arboviruses: Why today? One Heal. 2017;4(April):1–13.
5. ChristovaVa I, Gladnishka T, Taseva E, Kalvatchev N, Tsergouli K, Papa A. Seroprevalence of Crimean-Congo hemorrhagic fever virus, Bulgaria. Emerg Infect Dis. 2013;19(1):177–9.
6. Reads C. Barry Atkinson thesis The Characterisation of Arboviral Zoonoses in Central Asia. 2016;(September).
7. Atkinson B, Hewson R. Emerging arboviruses of clinical importance in Central Asia. J Gen Virol. 2018;99(9):1172–84.
8. Temirbekov J.T. Tick-borne encephalitis in Kazakhstan.pdf. 1985. p. 94–130.
9. Zlobin VI, Pogodina V V., Kahl O. A brief history of the discovery of tick-borne encephalitis virus in the late 1930s (based on reminiscences of members of the expeditions, their colleagues, and relatives). Ticks Tick Borne Dis [Internet]. 2017;8(6):813–20. Available from: http://dx.doi.org/10.1016/j.ttbdis.2017.05.001
10. Grascenkov NI. Tick-Borne Encephalitis in the Ussr. Bull World Health Organ. 1964;30:187–96.
11. Shapoval A.N. Tick-borne encephalitis. Ulickiy L.A., editor. Moscow: Medicina; 1980. 256 p.
12. Shapoval A.N., Ustinova A.P., Kipriyanova N.V., Shamarina A.G., Minayeva V.M., Lusin P.M., Golofastova E.E. SFY. The conclusion of the Tick borne encephalitis control in Perm Region.pdf. Perm region: The Tick-borne encephalitis in the West Ural region. The Perm State Medical University , the Health Care ministry of USSR; 1977. p. 4–7.
13. Dobler G. Zoonotic tick-borne flaviviruses. Vet Microbiol [Internet]. 2010 Jan 27 [cited 2011 Jun 18];140(3–4):221–8. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/19765917

14. Fritz R, Orlinger KK, Hofmeister Y, Janecki K, Traweger A, Perez-Burgos L, et al. Quantitative comparison of the cross-protection induced by tick-borne encephalitis virus vaccines based on European and Far Eastern virus subtypes. Vaccine [Internet]. 2012;30(6):1165–9. Available from: http://dx.doi.org/10.1016/j.vaccine.2011.12.013
15. Chao LH, Klein DE, Schmidt AG, Peña JM, Harrison SC. Sequential conformational rearrangements in flavivirus membrane fusion. Elife. 2014;3:e04389.
16. Hayasaka D, Suzuki Y, Kariwa H, Ivanov L, Volkov V, Demenev V, et al. Phylogenetic and virulence analysis of tick-borne encephalitis viruses from Japan and far-Eastern Russia. J Gen Virol [Internet]. 1999 Dec;80 (Pt 12:3127–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10567643
17. Asghar N, Lee YP, Nilsson E, Lindqvist R, Melik W, Kröger A, et al. The role of the poly(A) tract in the replication and virulence of tick-borne encephalitis virus. Sci Rep [Internet]. 2016;6(November):1–13. Available from: http://dx.doi.org/10.1038/srep39265
18. Kovalev SY, Mukhacheva TA. Reconsidering the classification of tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. Infect Genet Evol [Internet]. 2017;55:159–65. Available from: http://dx.doi.org/10.1016/j.meegid.2017.09.014
19. Zlobin V.I., Belikov S.I., Djioyev Yu.P., Kozlova I.V., Demina T.V. VMM et al. The Molecular episemiology of tick-borne encephalitis.pdf. In Irkutsk; 2002.
20. Kaiser R. Tick-Borne Encephalitis. Infect Dis Clin North Am. 2008;22(3):561–75.
21. Bourée P, Delaigue S, Ensaf A. Tick-borne encephalitis. Med Ther Pediatr. 2015;882–90.
22. Bogovic P. Tick-borne encephalitis: A review of epidemiology, clinical characteristics, and management. World J Clin Cases [Internet]. 2015;3(5):430. Available from: http://www.wjgnet.com/2307-8960/full/v3/i5/430.htm
23. Bogovic P, Lotric-Furlan S, Strle F. What tick-borne encephalitis may look like: Clinical signs and symptoms. Travel Med Infect Dis [Internet]. 2010;8(4):246–50. Available from: http://dx.doi.org/10.1016/j.tmaid.2010.05.011
24. LotriČ-Furlan S, Strle F. Thrombocytopenia, Leukopenia and abnormal liver function tests in the initial phase of Tick-borne Encephalitis. Zentralblatt fur Bakteriol. 1995;282(3):275–8.

25. Bourée P, Delaigue S, Ensaf A. Tick-borne encephalitis. Med Ther Pediatr. 2015;57:129–46.
26. Ierusalimsky A.P. Tick-borne encephalitis. In Novosibirsk: Novosibirsk, State medical academy publisher; 2001. p. 360.
27. Lindquist L, Vapalahti O. Tick-borne encephalitis. Lancet (London, England) [Internet]. 2008;371(9627):1861–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18514730
28. Kaiser R. The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994-98. A prospective study of 656 patients. Brain. 1999;122(11):2067–78.
29. Gritsun T, Frolova T, Zhankov A, Armesto M, Turner S, Frolova M, et al. Characterization of a siberian virus isolated from a patient with progressive chronic tick-borne encephalitis. J Virol. 2003;77(1):25–36.
30. Donoso Mantke O, Escadafal C, Niedrig M, Pfeffer M. Tick-borne encephalitis in Europe, 2007 to 2009. Eurosurveillance. 2011;16(39).
31. Haglund M, Günther G. Tick-borne encephalitis - Pathogenesis, clinical course and long-term follow-up. Vaccine. 2003;21(SUPPL. 1):2–9.
32. Mickienė A, Laiškonis A, Günther G, Vene S, Lundkvist Å, Lindquist L. Tickborne Encephalitis in an Area of High Endemicity in Lithuania: Disease Severity and Long- Term Prognosis. Clin Infect Dis [Internet]. 2002;35(6):650–8. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/342059
33. Bao ST. Prevention : Vaccines + Immunoglobulins. 2009;
34. Zaharcheva Tatyana. Tick-borne encephalitis in Habarovsk (Far-Eastern region) of Russian Federation. Scientific. 2013. 248 p.
35. Růžek D, Dobler G, Niller HH. May early intervention with high dose intravenous immunoglobulin pose a potentially successful treatment for severe cases of tick-borne encephalitis? BMC Infect Dis [Internet]. 2013;13(1):1. Available from: BMC Infectious Diseases
36. Morozova O V., Bakhvalova VN, Potapova OF, Grishechkin AE, Isaeva EI, Aldarov K V., et al. Evaluation of immune response and protective effect of four vaccines against the tick-borne encephalitis virus. Vaccine [Internet]. 2014;32(25):3101–6. Available from: http://dx.doi.org/10.1016/j.vaccine.2014.02.046
37. Kunz C. TBE vaccination and the Austrian experience. Vaccine. 2003;21(SUPPL. 1):50–5.

38. Lu Z, Bröker M, Liang G. Tick-Borne Encephalitis in Mainland China. Vector-Borne Zoonotic Dis [Internet]. 2008;8(5):713–20. Available from: http://www.liebertonline.com/doi/abs/10.1089/vbz.2008.0028
39. Chernokhaeva LL, Rogova Y V., Kozlovskaya LI, Romanova LI, Osolodkin DI, Vorovitch MF, et al. Experimental evaluation of the protective efficacy of tick-borne encephalitis (TBE) vaccines based on European and Far-Eastern TBEV strains in mice and in vitro. Front Microbiol. 2018;9(JUL):1–12.
40. Ershova AS, Gra OA, Lyaschuk AM, Grunina TM, Tkachuk AP, Bartov MS, et al. Recombinant domains III of Tick-Borne Encephalitis Virus envelope protein in combination with dextran and CpGs induce immune response and partial protectiveness against TBE virus infection in mice. BMC Infect Dis [Internet]. 2016;16(1):1–11. Available from: http://dx.doi.org/10.1186/s12879-016-1884-5
41. Blow JA, Dohm DJ, Negley DL, Mores CN. Virus inactivation by nucleic acid extraction reagents. J Virol Methods. 2004;119(2):195–8.
42. Haddock E, Feldmann F. Effective Chemical Inactivation of Ebola Virus. 2016;22(7).
43. Weidmann M, Avsic-Zupanc T, Bino S, Bouloy M, Burt F, Chinikar S, et al. Biosafety standards for working with crimean-congo hemorrhagic fever virus. J Gen Virol. 2016;97(11):2799–808.
44. Laboratory biosafety manual Third edition World Health Organization. 2004;
45. Veje M, Studahl M, Johansson M, Johansson P, Nolskog P. Diagnosing tick-borne encephalitis : a re-evaluation of notified cases. 2018;339–44.
46. Zlobin, V., Rudakov, N., Malov I. Tick-borne infections. Novosibirsk: Nauka; 2015.
47. Schwaiger M, Cassinotti P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. J Clin Virol. 2003;27(2):136–45.
48. Ackermann-Gäumann R, Tritten ML, Hassan M, Lienhard R. Comparison of three commercial IgG and IgM ELISA kits for the detection of tick-borne encephalitis virus antibodies. Ticks Tick Borne Dis [Internet]. 2018;9(4):956–62. Available from: http://dx.doi.org/10.1016/j.ttbdis.2018.03.031
49. Dobler G, Treib J, Kiessig ST, Von Blohn W, Frosner G, Haass A. Diagnosis of tick-borne encephalitis: Evaluation of sera with borderline titers with the TBE-ELISA. Infection. 1996;24(5):405–6.
50. Niedrig M, Avˇ T, Aberle SW. Quality control assessment for the serological diagnosis of tick borne encephalitis virus infections. 2007;38:260–4.

51. Amicizia D, Alexander Domnich, Donatella Panatto, Piero Luigi Lai, Maria Luisa Cristina UA and RG. Epidemiology of tick-borne encephalitis (TBE) in Europe and its prevention by available vaccines. Hum Vaccines Immunother [Internet]. 2013;(May):1163–71. Available from: http://widgets.ebscohost.com/prod/customlink/hanapi/hanapi.php?profile=4dfs1q6ik%2BHE5pTp1ZLu0eGT1tTR28jqzaLXyajL2dTV482lyNzZoJmu5NS8q%2BnpkKU%3D&DestinationURL=http%3A%2F%2Fsearch.ebscohost.com%2Flogin.aspx%3Fdirect%3Dtrue%26db%3Dbsu%26AN%3D94439330%26si
52. Süss J. Tick-borne encephalitis 2010: epidemiology, risk areas, and virus strains in Europe and Asia - An overview. Ticks Tick Borne Dis [Internet]. 2011;2(1):2–15. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1877959X10000956%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/21771531
53. Zlobin VI. Tick-borne encephalitis in the Russian Federation: state-of-the-art and prevention policy. Probl Virol. 2005;50(3):26–32.
54. Muto M, Bazartseren B, Tsevel B, Dashzevge E, Yoshii K, Kariwa H. Isolation and characterization of tick-borne encephalitis virus from Ixodes persulcatus in Mongolia in 2012. Ticks Tick Borne Dis [Internet]. 2015;6(5):623–9. Available from: http://dx.doi.org/10.1016/j.ttbdis.2015.05.006
55. Golovljova I, Vene S, Sjölander KB, Vasilenko V, Plyusnin A, Lundkvist Å. Characterization of tick-borne encephalitis virus from Estonia. J Med Virol. 2004;74(4):580–8.
56. Tikunova N V. Ixodes persulacatus/I.pavlovskyi natural hybrids:occurence and infection by a wide range of tick-transmitted agents. In: Journal of infectious pathology. 2018. p. 82–3.
57. Hasle G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. 2013;3(September):1–6.
58. Dmitrovskiy A. TBE in Kazakhstan. In: Gerhard Dobler, Wilhelm, Erber H-JS, editor. TBE-The Book [Internet]. Global health press; 2016. Available from: idea.org/tbe/tbe-countries-kazakhstan
59. Linetskaya Yu. The strains of spring summer encephalitis in Almaty oblast. Kazakh state Medical University named by V.M.Molotov (currently Kazakh National Medical University named S.D. Asfendiarov); 1949.
60. L’vov DK, Al’khovskiĭ SV, Shchelkanov MIu, Deriabin PG, Gitel’man AK, Botikov AG AV. Genetic characterisation of Powassan virus (POWV) isolated from

Haemophysalis longicornis ticks in Primorye and two strains of Tick-borne encephalitis virus (TBEV) (Flaviviridae, Flavivirus): Alma-Arasan virus (AAV) isolated from Ixodes persulcatus ticks . Probl Virol. 2014;59(5):18–22.

61. Atshabar B., Burdenov L. A V. Atlas of Bacterial and Virus Zoonotic Infections Distribution in Kazakhstan. L.A. B, editor. Almaty: Kazakh Scientific Center of Quarantine and Zoonotic diseases; 2012. 232 p.
62. Kirushenko T.V., Drobishenko N.I., Lvov D.K., Rogovaya S.G. KSK. The hosts and vectors of arboviral infectious in Ili-karatal foci. In: Karimov S, editor. The virus ecology in Kazakhstan and in Central Asia. Alma-ata: Karimov, S.; 1980. p. 64–73.
63. Karimov S.K., Dernovoy A.G. DEE. Arbovirues and arboviral diseases in the Republic of Kazakhstan. Ismagulov, A.T. SOS, editor. Almaty; 2001. 162 p.
64. Nakayasu M, Hirano M, Muto M, Kobayashi S, Kariwa H, Yoshii K. Development of a serodiagnostic IgM-ELISA for tick-borne encephalitis virus using subviral particles with strep-tag. Ticks Tick Borne Dis [Internet]. 2018;9(6):1391–4. Available from: https://doi.org/10.1016/j.ttbdis.2018.06.010
65. Whitehouse C a. Crimean-Congo hemorrhagic fever. In: Antiviral Research. 2004. p. 145–60.
66. Cherenov I. The features of CCHF clinical presence . CCHF diagnostics and treatment. Astrahan Medical Academy; 2006.
67. Merl J, Asia FIN, Casals D, Henderson BE, Karl M, Wiebenga NH, et al. THE EPIDEMIOLOGY OF TICK-BORNE CRIMEAN-CONGO HEMORRHAGIC. 1979;
68. Honig JE, Osborne JC, Nichol ST. Crimean – Congo hemorrhagic fever virus genome L RNA segment and encoded protein. 2004;321:29–35.
69. Bente DA, Forrester NL, Watts DM, McAuley AJ, Whitehouse CA, Bray M. Crimean-Congo hemorrhagic fever: History, epidemiology, pathogenesis, clinical syndrome and genetic diversity. Antiviral Res [Internet]. 2013;100(1):159–89. Available from: http://dx.doi.org/10.1016/j.antiviral.2013.07.006
70. Holm T, Kopicki J-D, Busch C, Olschewski S, Rosenthal M, Uetrecht C, et al. Biochemical and structural studies reveal differences and commonalities among cap-snatching endonucleases from segmented negative-strand RNA viruses. J Biol Chem [Internet]. 2018;293:jbc.RA118.004373. Available from: http://www.jbc.org/lookup/doi/10.1074/jbc.RA118.004373
71. Bergeron E, Vincent MJ, Nichol ST. Crimean-Congo Hemorrhagic Fever Virus Glycoprotein Processing by the Endoprotease SKI-1/S1P Is Critical for Virus

Infectivity. J Virol [Internet]. 2007;81(23):13271–6. Available from: http://jvi.asm.org/cgi/doi/10.1128/JVI.01647-07

72. Bertolotti-Ciarlet A, Smith J, Strecker K, Paragas J, Altamura LA, McFalls JM, et al. Cellular Localization and Antigenic Characterization of Crimean-Congo Hemorrhagic Fever Virus Glycoproteins. J Virol [Internet]. 2005;79(10):6152–61. Available from: http://jvi.asm.org/cgi/doi/10.1128/JVI.79.10.6152-6161.2005
73. Sara S, Bokaean M, Shahrivar MR. Crimean-Congo Hemorrhagic Fever. Lab Med. 2015;46(3):180–9.
74. Vassilenko SM, Vassilev TL, Bozadjiev LG, Bineva IL, Kazarov GZ. Specific intravenous immunoglobulin for Crimean-Congo haemorrhagic fever. Lancet. 1990;335(8692):791–2.
75. Ergönül Ö. Crimean-Congo haemorrhagic fever. Lancet Infect Dis [Internet]. 2006;6(4):203–14. Available from: http://www.sciencedirect.com/science/article/B6W8X-4JHV0JG-N/1/f7685e05971b2796f34bdc5f218facf2
76. Hewson R, Chamberlain J, Mioulet V, Lloyd G, Jamil B, Hasan R, et al. Crimean-Congo haemorrhagic fever virus : sequence analysis of the small RNA segments from a collection of viruses world wide □. 2004;102:185–9.
77. Chinikar S, Bouzari S, Shokrgozar MA, Mostafavi E. Genetic Diversity of Crimean Congo Hemorrhagic Fever Virus Strains from Iran. 2016;10(June):127–40.
78. Hewson R, Gmyl A, Gmyl L, Smirnova SE, Karganova G, Jamil B, et al. Evidence of segment reassortment in Crimean-Congo haemorrhagic fever virus. J Gen Virol [Internet]. 2004 Oct [cited 2014 Jun 7];85(Pt 10):3059–70. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15448369
79. Ergonul O. Treatment of Crimean-Congo hemorrhagic fever. Antiviral Res. 2008;78:125–31.
80. Morikawa S, Saijo M, Kurane I. Recent progress in molecular biology of Crimean-Congo hemorrhagic fever. Comp Immunol Microbiol Infect Dis. 2007;30:375–89.
81. Mishra B, Appannanavar S. An update on crimean congo hemorrhagic fever. J Glob Infect Dis. 2011;3(3):285.
82. Swanepoel AR, Gill DE, Shepherd AJ, Leman PA, Mynhardt JH. The Clinical Pathology of Crimean-Congo Hemorrhagic Fever Source : Reviews of Infectious Diseases , Vol . 11 , Supplement 4 . Hemostatic Impairment Associated with Hemorrhagic Fever Viruses (May - Jun ., 1989), pp . S794-S800 Published by : Oxford Unive. 2016;11.

83. Cevik M a, Erbay A, Bodur H, Gülderen E, Baştuğ A, Kubar A, et al. Clinical and laboratory features of Crimean-Congo hemorrhagic fever: predictors of fatality. Int J Infect Dis [Internet]. 2008;12(4):374–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18063402
84. Sargianou M, Panos G, Tsatsaris A, Gogos C, Papa A. Crimean-Congo hemorrhagic fever: Seroprevalence and risk factors among humans in Achaia, western Greece. Int J Infect Dis [Internet]. 2013;17(12):e1160–5. Available from: http://dx.doi.org/10.1016/j.ijid.2013.07.015
85. Akinci E, Bodur H, Sunbul M, Leblebicioglu H. Prognostic factors, pathophysiology and novel biomarkers in Crimean-Congo hemorrhagic fever. Antiviral Res [Internet]. 2016;132:233–43. Available from: http://dx.doi.org/10.1016/j.antiviral.2016.06.011
86. Ergunay K, Kocak Tufan Z, Bulut C, Kinikli S, Demiroz AP, Ozkul A. Antibody responses and viral load in patients with Crimean-Congo hemorrhagic fever: a comprehensive analysis during the early stages of the infection. Diagn Microbiol Infect Dis [Internet]. 2014;79(1):31–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0732889314000054
87. Mardani M, Jahromi MK, Naieni KH, Zeinali M. The Efficacy of Oral Ribavirin in the Treatment of Crimean-Congo Hemorrhagic Fever in Iran. Clin Infect Dis [Internet]. 2003;36(12):1613–8. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/375058
88. Mendoza EJ, Warner B, Safronetz D, Ranadheera C. Crimean–Congo haemorrhagic fever virus: Past, present and future insights for animal modelling and medical countermeasures. Zoonoses Public Health. 2018;65(5):465–80.
89. Johnson S, Maayan N, Mills I, Buckley B, Kakourou A, R. M, et al. Ribavirin for treating Crimean Congo haemorrhagic fever. Cochrane Database Syst Rev [Internet]. 2017;2017(7). Available from: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L617221473%0Ahttp://dx.doi.org/10.1002/14651858.CD012713%0Ahttps://www.scopus.com/inward/record.uri?eid=2-s2.0-85022192927&doi=10.1002%2F14651858.CD012713&partnerID=40&md5=43fc7b823d
90. Andersson I, Bladh L, Mousavi-jazi M, Magnusson K, Haller O, Mirazimi A, et al. Andersson-2004-Human MxA protein inhibits the. 2004;78(8):4323–9.
91. Xue F, Zhu L, Liu S, Liu W, Yang C, Wang L, et al. Long noncoding RNA

ADAMTS9-AS2 is regulated by DNA methyltransferase 1 and inhibits the malignant behaviors of non-small cell lung cancer cells. Int J Clin Exp Pathol. 2017;10(3):2599–608.

92. Bowick GC, Airo AM, Bente DA. Expression of interferon-induced antiviral genes is delayed in a STAT1 knockout mouse model of Crimean-Congo hemorrhagic fever. Virol J [Internet]. 2012;9(1):1. Available from: ???
93. Leblebicioglu H, Bodur H, Dokuzoguz B, Elaldi N, Guner R, Koksal I, et al. Case Management and Supportive Treatment for Patients with Crimean-Congo Hemorrhagic Fever. Vector-Borne Zoonotic Dis [Internet]. 2012;12(9):805–11. Available from: http://online.liebertpub.com/doi/abs/10.1089/vbz.2011.0896
94. Dowall SD, Carroll MW, Hewson R. Development of vaccines against Crimean-Congo haemorrhagic fever virus. Vaccine [Internet]. 2017;35(44):6015–23. Available from: https://doi.org/10.1016/j.vaccine.2017.05.031
95. Hawman DW, Feldmann H. Open Peer Review Recent advances in understanding Crimean-Congo hemorrhagic fever virus [version 1; referees: 4 approved]. 2018;7(0):1–10. Available from: https://doi.org/10.12688/f1000research.16189.1
96. Papa A, Papadimitriou E. The Bulgarian vaccine Crimean-Congo haemorrhagic fever virus strain. 2011;(November 2010):225–9.
97. Garrison AR, Shoemaker CJ, Golden JW, Fitzpatrick CJ, Suschak JJ, Richards MJ, et al. A DNA vaccine for Crimean-Congo hemorrhagic fever protects against disease and death in two lethal mouse models. PLoS Negl Trop Dis. 2017;11(9):1–19.
98. Capsid FV, Protection I, Mice C. crossm Immunization with DNA Plasmids Coding for Crimean-Congo Hemorrhagic Fever Virus Capsid and Envelope Proteins and / or Virus-Like Particles. 2017;91(10):1–19.
99. Dowall SD, Buttigieg KR, Findlay-Wilson SJD, Rayner E, Pearson G, Miloszewska A, et al. A Crimean-Congo hemorrhagic fever (CCHF) viral vaccine expressing nucleoprotein is immunogenic but fails to confer protection against lethal disease. Hum Vaccines Immunother. 2016;12(2):519–27.
100. Buttigieg KR, Dowall SD, Findlay-Wilson S, Miloszewska A, Rayner E, Hewson R, et al. A novel vaccine against Crimean-Congo haemorrhagic fever protects 100% of animals against lethal challenge in a mouse model. PLoS One. 2014;9(3).
101. Ghiasi SM, Salmanian AH, Chinikar S, Zakeri S. Mice orally immunized with a transgenic plant expressing the glycoprotein of Crimean-Congo hemorrhagic fever

virus. Clin Vaccine Immunol. 2011;18(12):2031–7.

102. Canakoglu N, Berber E, Tonbak S, Ertek M, Sozdutmaz I, Aktas M, et al. Immunization of Knock-Out α/β Interferon Receptor Mice against High Lethal Dose of Crimean-Congo Hemorrhagic Fever Virus with a Cell Culture Based Vaccine. PLoS Negl Trop Dis. 2015;9(3):1–14.
103. Chosewood L.Casey WED, editor. Biosafety in Microbiological and Biomedical Laboratories. 5th ed. U.S. Department of Health and Human Services; 2009.
104. Leblebicioglu H, Ozaras R, Sunbul M. American Journal of Infection Control Crimean-Congo hemorrhagic fever : A neglected infectious disease with potential nosocomial infection threat Yellow fever outbreaks and Twitter : Rumors and misinformation. AJIC Am J Infect Control [Internet]. 2017;45(7):815–6. Available from: http://dx.doi.org/10.1016/j.ajic.2016.05.039
105. Remy MM, Alfter M, Chiem MN, Barbani MT, Engler OB, Suter-Riniker F. Effective chemical virus inactivation of patient serum compatible with accurate serodiagnosis of infections. Clin Microbiol Infect [Internet]. 2018; Available from: https://doi.org/10.1016/j.cmi.2018.10.016
106. Atkinson B, Chamberlain J, Logue CH, Cook N, Bruce C, Dowall SD, et al. Development of a Real-Time RT-PCR Assay for the Detection of Crimean-Congo Hemorrhagic Fever Virus. Vector-Borne Zoonotic Dis. 2012;12(9):786–93.
107. Rosenstierne MW, Karlberg H, Bragstad K, Lindegren G, Stoltz ML, Salata C, et al. Rapid bedside inactivation of Ebola virus for safe nucleic acid tests. J Clin Microbiol. 2016;54(10):2521–9.
108. Papa A, Mirazimi A, Köksal I, Estrada-Pena A, Feldmann H. Recent advances in research on Crimean-Congo hemorrhagic fever. J Clin Virol [Internet]. 2015;64:137–43. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1386653214003680
109. Yapar M, Aydogan H, Pahsa A, Besirbellioglu BA, Bodour H, Basustaoglu AC, et al. Rapid and quantitative detection of Crimean-Congo hemorrhagic fever virus by one-step real-time reverse transcriptase-PCR. Jpn J Infect Dis. 2005;58(6):358–62.
110. Jääskeläinen AJ, Kallio-Kokko H, Ozkul A, Bodur H, Korukruoglu G, Mousavi M, et al. Development and evaluation of a real-time RT-qPCR for detection of Crimean-Congo hemorrhagic fever virus representing different genotypes. Vector Borne Zoonotic Dis [Internet]. 2014;14(12):870–2. Available from: http://online.liebertpub.com/doi/abs/10.1089/vbz.2014.1577

111. Zahraei BH, Hashem Zadeh MS, Najar Asl M, Zahiri Yeganeh S, Tat M, Metanat M, et al. Novel, In-House, SYBR Green Based One-Step rRT-PCR: Rapid and Accurate Diagnosis of Crimean-Congo Hemorrhagic Fever Virus in Suspected Patients From Iran. Jundishapur J Microbiol [Internet]. 2016;9(1):2–6. Available from: http://www.jjmicrobiol.com/?page=article&article_id=29246
112. Wölfel R, Paweska JT, Petersen N, Grobbelaar AA, Leman PA, Hewson R, et al. Low-density macroarray for rapid detection and identification of Crimean-Congo hemorrhagic fever virus. J Clin Microbiol. 2009;47(4):1025–30.
113. Vanhomwegen J. First multicentre evaluation of serological and molecular diagnostic assays for Crimean-Congo hemorrhagic fever J. 2012;3:15–6.
114. Osman HAM, Eltom KH, Musa NO, Bilal NM, Elbashir MI, Aradaib IE. Development and evaluation of loop-mediated isothermal amplification assay for detection of Crimean Congo hemorrhagic fever virus in Sudan. J Virol Methods [Internet]. 2013;190(1–2):4–10. Available from: http://dx.doi.org/10.1016/j.jviromet.2013.03.004
115. Ke R, Zorzet A, Göransson J, Lindegren G, Sharifi-Mood B, Chinikar S, et al. Colorimetric nucleic acid testing assay for RNA virus detection based on circle-to-circle amplification of padlock probes. J Clin Microbiol. 2011;49(12):4279–85.
116. Burt FJ, Leman PA, Abbott JC, Swanepoel R. Serodiagnosis of Crimean-Congo haemorrhagic fever. Epidemiol Infect [Internet]. 1994;113(3):551–62. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2271329&tool=pmcentrez&rendertype=abstract
117. Leader WG. Brief Report of tele-conference of the CCHF Working Group. 2009;
118. Whitehouse CA. Crimean-Congo hemorrhagic fever. Antiviral Res. 2004;64(3):145–60.
119. Lwande OW, Irura Z, Tigoi C, Chepkorir E, Orindi B, Musila L, et al. Seroprevalence of Crimean Congo Hemorrhagic Fever Virus in Ijara District, Kenya. Vector-Borne Zoonotic Dis. 2012;12(9):727–32.
120. Pshenichnaya NY, Sydenko IS, Klinovaya EP, Romanova EB, Zhuravlev AS. Possible sexual transmission of Crimean-Congo hemorrhagic fever. Int J Infect Dis [Internet]. 2016;45:109–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26972040
121. Sharifinia N, Rafinejad J, Hanafi Bojd AAI, Chinikar S, Piazak N, Baniardalan M, et al. Hard Ticks (Ixodidae) and Crimean-Congo Hemorrhagic Fever Virus in

South West of Iran. Acta Med Iran [Internet]. 2015;53(3):177–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25796025

122. Telmadarraiy Z, Chinikar S, Vatandoost H, Faghihi F, Hosseini-Chegeni A. Vectors of Crimean Congo Hemorrhagic Fever virus in Iran. J Arthropod Borne Dis. 2015;9(2):137–47.
123. Апанаскевич ДА, Филиппова НА. К идентификации видов и подвидов рода Hyalomma (ACARI : IXODIDAE) фауны России и сопредельных территорий по личиночной фазе. 2007;268–83.[Species identification of Hyalomma (ACARI:IXODIDAE) in Russian and bordering territories], in rissian.
124. Умирзакова А., Оралханова М. СЗ. К фауне иксодовых клещей (Acariformes, Ixodoidea) Шиелийского района Кызылординской области.pdf. Al-Farabi Kazakh Natl Univ Bull Ecol Ser. 2015;44:775–7.[About the fauna of Ixodae, in Shiely region of Kyzylorda oblast], in russian.
125. Klaus C, Gethmann J, Hoffmann B, Ziegler U, Heller M, Beer M. Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany. Parasitol Res [Internet]. 2016;115(7):2729–40. Available from: http://dx.doi.org/10.1007/s00436-016-5022-5
126. Messina JP, Pigott DM, Golding N, Duda KA, Brownstein JS, Weiss DJ, et al. The global distribution of Crimean-Congo hemorrhagic fever. Trans R Soc Trop Med Hyg. 2015;109(8):503–13.
127. Nurmakhanov T, Sansyzbaev Y, Atshabar B, Deryabin P, Kazakov S, Zholshorinov A, et al. Crimean-Congo haemorrhagic fever virus in Kazakhstan (1948-2013). Int J Infect Dis [Internet]. 2015;38:19–23. Available from: http://dx.doi.org/10.1016/j.ijid.2015.07.007
128. Abdikarimov M. Crimean-Congo Hemorrhagic Fever. In: Beren. Almaty; 1995. p. 65. In russian.
129. Kupča AM, Essbauer S, Zoeller G, de Mendonça PG, Brey R, Rinder M, et al. Isolation and molecular characterization of a tick-borne encephalitis virus strain from a new tick-borne encephalitis focus with severe cases in Bavaria, Germany. Ticks Tick Borne Dis [Internet]. 2010 Mar [cited 2011 Jul 15];1(1):44–51. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1877959X09000041
130. Wölfel R, Paweska JT, Petersen N, Grobbelaar AA, Leman PA, Hewson R, et al. Virus detection and monitoring of viral load in Crimean-Congo hemorrhagic fever virus patients. Emerg Infect Dis. 2007;13(7):1097–100.
131. Zhang Z, Schwartz S, Wagner L, Miller W. A Greedy Algorithm for Aligning DNA

Sequences. J Comput Biol [Internet]. 2000;7(1–2):203–14. Available from: http://www.liebertonline.com/doi/abs/10.1089/10665270050081478

132. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. Bioinformatics. 2008;24(16):1757–64.
133. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.
134. Charan J, Biswas T. How to calculate sample size for different study designs in medical research? Indian J Psychol Med [Internet]. 2013;35(2):121. Available from: http://www.ijpm.info/text.asp?2013/35/2/121/116232
135. Altman DG. Practical statistics for medical research. Chapman an. Vol. 91. London; 2017. 399-404 p.
136. Weidong Gua, Thomas R. Unnascha, Charles R. Katholib, Richard Lampmanc and RJN. Fundamental issues in mosquito surveillance for arboviral transmission. Trans R Soc Trop Med Hyg. 2008;8(8):817–22.
137. Gu W, Lampman R, Novak RJ. Problems in Estimating Mosquito Infection Rates Using Minimum Infection Rate. J Med Entomol [Internet]. 2003;40(5):595–6. Available from: https://academic.oup.com/jme/article-lookup/doi/10.1603/0022-2585-40.5.595
138. Morikawa S, Saijo M, Kurane I. Recent progress in molecular biology of Crimean-Congo hemorrhagic fever. Comp Immunol Microbiol Infect Dis. 2007;30(5–6):375–89.
139. Kalmakova M.A., Matzhanova A.M., Sayakova Z.Z., Bodikov M.Z. IBG. About fauna of ixodid ticks -vectors in natural foci infectious diseases. In: Problems of saving the biodeversity in Kazakhstan and bordering countries in nature and collections. Almaty: Kazakh National Universtity named by Al-Farabi; 2016. p. 88–91. In russian.
140. Leonovich S.A., Fine structural features of the sensory system in the ixodid tick dermacentor niveus (Parasitiformes, Ixodidae). Parazitologia. 2009;43(1):3–11.
141. Sayakova Z. To the fauna of ixodid ticks (Acari, Ixodidae) of Ile-Alatau national park.pdf. 2014.
142. Романенко Н. Эколого-этологические аспекты изучения иксодовых клещей (Parasitiformes, Ixodae) различных ландшафтов. Vol. 67. 2007. [Ecological aspects of Ixodae], in russian.
143. Yoshii K, Song JY, Park SB, Yang J, Schmitt HJ. Tick-borne encephalitis in

Japan, Republic of Korea and China. Emerg Microbes Infect [Internet]. 2017;6(9):e82. Available from: http://dx.doi.org/10.1038/emi.2017.69

144. Shapiyeva Z, Tolemisova A, Zhandosov S, Yegemberdiyeva R ZL. Identification of Tick-Borne Encephalitis virus in ticks from Northern Kazakhstan. In Wasington: American society for microbilogy; 2014. p. 59.
145. Dobler G, Zoller G, Poponnikova T, Pfeffer M, Essbauer S. Tick-borne encephalitis virus in a highly endemic area in Kemerovo (Western Siberia, Russia). Int J Med Microbiol. 2008;298:94–101.
146. Frey S, Mossbrugger I, Altantuul D, Battsetseg J, Davaadorj R, Tserennorov D, et al. Isolation, preliminary characterization, and full-genome analyses of tick-borne encephalitis virus from Mongolia. Virus Genes [Internet]. 2012 Dec [cited 2013 Mar 19];45(3):413–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22847274
147. Donoso Mantke O, Aberle SW, Avsic-Zupanc T, Labuda M, Niedrig M. Quality control assessment for the PCR diagnosis of tick-borne encephalitis virus infections. J Clin Virol [Internet]. 2007 Jan [cited 2014 Jan 18];38(1):73–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17070730
148. Briggs BJ, Atkinson B, Czechowski DM, Larsen PA, Meeks HN, Carrera JP, et al. Tick-Borne Encephalitis Virus, Kyrgyzstan. 2011;17(5):876–9.
149. Kovshar A.V. Ornithofauna revision and modern list of birds of Kazakhstan. 2012;9(574):51–70.
150. Ryabitsev, Vadim, Kovshar, Anatoliy, Kovshar, Victoria, Berezovikov N. A field guide to the birds of Kazakhstan. 2014. 512 p.
151. Kovalev SY, Chernykh DN, Kokorev VS, Snitkovskaya TE, Romanenko V V. Origin and distribution of tick-borne encephalitis virus strains of the Siberian subtype in the Middle Urals, the north-west of Russia and the Baltic countries. J Gen Virol. 2009;90:2884–92.
152. Waldenström J, Lundkvist Å, Falk KI, Garpmo U, Bergström S, Lindegren G, et al. Migrating birds and tickborne encephalitis virus. Emerg Infect Dis. 2007;13(8):1215–8.
153. Mikryukova TP, Moskvitina NS, Kononova Y V., Korobitsyn IG, Kartashov MY, Tyuten'kov OY, et al. Surveillance of tick-borne encephalitis virus in wild birds and ticks in Tomsk city and its suburbs (Western Siberia). Ticks Tick Borne Dis [Internet]. 2014;5(2):145–51. Available from: http://dx.doi.org/10.1016/j.ttbdis.2013.10.004

154. Weidmann M, Růžek D, Křivanec K, Zöller G, Essbauer S, Pfeffer M, et al. Relation of genetic phylogeny and geographical distance of tick-borne encephalitis virus in central europe. J Gen Virol. 2011;92(8):1906–16.
155. Sparagano O, George D, Giangaspero A, Špitalská E. Arthropods and associated arthropod-borne diseases transmitted by migrating birds. The case of ticks and tick-borne pathogens. Vet Parasitol. 2015;213(1–2):61–6.
156. Korenberg EI, Pchelkina AA, Spitsina LN. Consistent patterns in the contact of domestic animals with tick-borne encephalitis virus in the eastern part of the Russian plain. J Hyg Epidemiol Microbiol Immunol [Internet]. 1984;28(1):73–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6715878
157. Ergönül Ö. Crimean-Congo haemorrhagic fever. Lancet Infect Dis. 2006;6(April):203–14.
158. Christova I, Younan R, Taseva E, Gladnishka T, Trifonova I, Ivanova V, et al. Hemorrhagic Fever with Renal Syndrome and Crimean-Congo Hemorrhagic Fever as Causes of Acute Undifferentiated Febrile Illness in Bulgaria. Vector Borne Zoonotic Dis [Internet]. 2013;13(3):188–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23421884
159. Cunha BA, Lortholary O, Cunha CB. Fever of unknown origin: A clinical approach. Am J Med [Internet]. 2015;128(10):1138.e1-1138.e15. Available from: http://dx.doi.org/10.1016/j.amjmed.2015.06.001
160. Knust B, Medetov ZB, Kyraubayev KB, Bumburidi Y, Erickson BR, MacNeil A, et al. Crimean-Congo hemorrhagic fever, Kazakhstan, 2009-2010. Emerg Infect Dis. 2012;18(4):643–5.
161. Messina JP, Pigott DM, Duda KA, Brownstein JS, Myers MF, George DB, et al. A global compendium of human Crimean-Congo haemorrhagic fever virus occurrence. Sci data [Internet]. 2015;2:150016. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4409013&tool=pmcentrez&rendertype=abstract
162. Ertugrul B, Uyar Y, Yavas K, Turan C, Oncu S, Saylak O, et al. An outbreak of Crimean-Congo hemorrhagic fever in western Anatolia, Turkey. Int J Infect Dis. 2009;13(6).
163. Bodur H, Akinci E, Ascioglu S, ??ng??r?? P, Uyar Y. Subclinical infections with Crimean-Congo hemorrhagic fever virus, Turkey. Emerg Infect Dis. 2012;18(4):640–2.
164. Abdiyeva K, Turebekov N, Dmitrovsky A, Tukhanova N, Shin A, Yeraliyeva L, et

al. Seroepidemiological and molecular investigations of infections with Crimean–Congo haemorrhagic fever virus in Kazakhstan. Int J Infect Dis. 2019;78:121–7.

165. Izadi S, Holakouie-Naieni K, Majdzadeh SR, Chinikar S, Nadim A, Rakhshani F, et al. Seroprevalence of Crimean-Congo hemorrhagic fever in Sistan-va-Baluchestan Province of Iran. Jpn J Infect Dis. 2006;59(5):326–8.
166. Vanhomwegen J, Alves MJ, Zupanc TA, Bino S, Chinikar S, Karlberg H, et al. Diagnostic assays for Crimean-Congo hemorrhagic fever. Emerg Infect Dis [Internet]. 2012;18(12):1958–65. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3557897&tool=pmcentrez&rendertype=abstract
167. Fajs L, Humolli I, Saksida A, Knap N, Jelov??ek M, Korva M, et al. Prevalence of Crimean-Congo hemorrhagic fever virus in healthy population, livestock and ticks in Kosovo. PLoS One. 2014;9(11):8–10.
168. Jr VHMRHJLCC, Santos M. Skin manifestations of tick bites in humans *. An Bras Dermatol. 2018;93(Figure 3):251–5.
169. Cikman A, Aydin M, Gulhan B, Karakecili F, Kesik OA, Ozcicek A, et al. Seroprevalence of Crimean-Congo Hemorrhagic Fever Virus in Erzincan Province, Turkey, Relationship with Geographic Features and Risk Factors. Vector Borne Zoonotic Dis [Internet]. 2016;16(3):199–204. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26808904
170. Wasfi F, Dowall S, Ghabbari T, Bosworth A, Chakroun M, Varghese A, et al. Sero-epidemiological survey of Crimean-Congo hemorrhagic fever virus in Tunisia. Parasite [Internet]. 2016;23:10. Available from: http://www.parasite-journal.org/10.1051/parasite/2016010
171. Ertugrul B, Kirdar S, Ersoy OS, Ture M, Erol N, Ozturk B, et al. The seroprevalence of Crimean-Congo haemorrhagic fever among inhabitants living in the endemic regions of Western Anatolia. Scand J Infect Dis. 2012;44(June 2011):276–81.
172. Sidira P, Maltezou HC, Haidich AB, Papa A. Seroepidemiological study of Crimean-Congo haemorrhagic fever in Greece, 2009-2010. Clin Microbiol Infect [Internet]. 2012;18(2):E16–9. Available from: http://dx.doi.org/10.1111/j.1469-0691.2011.03718.x
173. Yashina L, Petrova I, Seregin S, Vyshemirskii O, Lvov D, Aristova V, et al. Genetic variability of Crimean-Congo haemorrhagic fever virus in Russia and Central Asia. J Gen Virol. 2003;84(5):1199–206.

174. Chinikar S, Shah-Hosseini N, Bouzari S, Shokrgozar MA, Mostafavi E, Jalali T, et al. Assessment of recombination in the s-segment genome of crimean-congo hemorrhagic fever virus in iran. J Arthropod Borne Dis. 2016;10(1):12–23.
175. Zhou Z, Deng F, Han N, Wang H, Sun S, Zhang Y, et al. Reassortment and migration analysis of Crimean-Congo haemorrhagic fever virus. J Gen Virol. 2013;94:2536–48.
176. Leblebicioglu H, Eroglu C, Erciyas-Yavuz K, Hokelek M, Acici M, Yilmaz H. Role of migratory birds in spreading Crimean-Congo hemorrhagic fever, Turkey. Emerg Infect Dis. 2014;20(8):1331–4.
177. Levin KA. Study design III: Cross-sectional studies. Evid Based Dent. 2006;7(1):24–5.

Annex 2. List of publications in International Peer-reviewed Journals

1. **Abdiyeva K**, Turebekov N, Dmitrovsky A, Tukhanova N, Shin A, Yeraliyeva L,Heinrich N, Hoelscher M, Yegemberdiyeva R, Shapiyeva Z, Kachiyeva Z,Zhalmagambetova A, Montag J, Dobler G, Zinner J, Wagner E, Frey S, Essbauer S. Seroepidemiological and molecular investigations of infections with Crimean-Congo haemorrhagic fever virus in Kazakhstan. Int J Infect Dis. 2019 Jan;78:121-127.
2. Khosa C, Patel K, **Abdiyeva K**, Turebekov N, Prüller B, Heinrich N. Proceedings from the CIHLMU 5th Infectious Diseases Symposium 2016 "Drug Resistant Tuberculosis: Old Disease - New Challenge". BMC Proc. 2017 Sep 4;11(Suppl 10):0. doi: 10.1186/s12919-017-0077-6.
3. Turebekov N, **Abdiyeva K**, Yegemberdiyeva R, Dmitrovsky A, Yeraliyeva L, Shapiyeva Z, Amirbekov A, Oradova A, Kachiyeva Z, Ziyadina L, Hoelscher M, Dobler G, Zinner J, Frey S, Essbauer S. Prevalence of Rickettsia species in ticks including identification of unknown species in two regions in Kazakhstan. Parasites&Vectors (accepted paper in April 2019)

Annex 3. Statement of Pre-release and contribution

Two articles are part of this project study. The international journal of infectious diseases published one of them. The title of this published article is "Seroepidemiological and molecular investigations of infections with Crimean-Congo hemorrhagic fever virus in Kazakhstan."

The second article titled “Epidemiological and Molecular investigations of TBEV in Kazakhstan give new insights in vectors and phylogeny” is being reviewed for the submission to Parasites and Vectors peer-reviewed journal.

The Ph.D. candidate Karlygash Abdiyeva collected the sera samples and ticks for further investigation herself and with the help of trained specialists of medical facilities of target oblasts and Scientific Practical Center of Sanitary-Epidemiological expertise and Monitoring (SPCSEEM). All laboratory work and evaluation of results were done by the Ph.D. candidate under the close supervision of the direct supervisor. Moreover, Ph.D. candidate Karlygash Abdiyeva did the data evaluation and analyzing, wrote two articles under the close supervision of all supervisor.

Annex 4. Acknowledgments

I want to express my gratitude to German-Kazakh network for biosafety and biosecurity in the framework of the German biosecurity Program financed by the Federal Ministry of Foreign Affairs of Germany for the opportunity to study and make science. My gratitude goes to the Institute of Microbiology of Bundeswehr, especially prof. Zoller, Joshua Zinner, Sabine Gallaher, Kerstin Roesel, Edith Wagner and Stefan Frey for their kind support during my studying and valuable inputs.

I am sincerely grateful to my direct supervisor Dr. Sandra Essbauer for her excellent supervision, her immense patience with me, given knowledge, support during the Ph.D. program. With her supervision, I could overcome all difficulties during the Ph.D. program. I thank my habilitated supervisor prof. Michael Hoelscher, my LMU supervisor Dr. Norbert Heinrich and local supervisor Dr.Gerhard Dobler for their supervision, support and opportunity to get brilliant knowledge at the CIH for LMU. I also thank the team of the CIH for LMU, especially Dr.Guenter Froeschl for his help and valuable tips.

I want to express my gratitude to GIZ office in Kazakhstan especially to Aliya Zhalmagambetova, Olga Kressova and GIZ office in Berlin to Olga Speiser and Lilian Hollenweger for their generous support.

I want to express my gratitude to specialists of the SPCEEM and Kazakh National Medical University (KNMU) for help and opportunity to make the science in their laboratories.

My gratitude goes to my family especially for my dearest mother Rahima Yebekenova who supported me in every awkward moment and to my father Smadi Abdiyev who encouraged me to be a scientist.

Annex 6. Informed Consent Form

“Study of Patients with Fever of unknown origin due to selected Infectious Diseases in five regions (oblasts) of Kazakhstan”

You are being invited to join this research study because we want to find out if you have an acute infection with certain bacteria and viruses. It is possible to do a blood test to see if you have been exposed to these infections, either recently or long ago. After a person is exposed to a virus or bacteria, they produce substances called antibodies. Antibodies are proteins made by the body's natural defense system (immune system) to attack and destroy foreign substances, such as bacteria and viruses.

These antibodies can remain in the blood of people for many years after exposure to the infection. This study is designed to measure antibodies against some infections.

The infections to be studied include brucellosis, leptospirosis, leishmaniosis, meloidosis, glanders, borreliosis, erlichiosis, anaplasmosis, Q-fever, spotted fever, plague, anthrax, tularaemia,typhus group and scrub typhus group rickettsioses, tick-borne encephalitis (TBE), West Nile fever, Dengue virus infections, Japanese encephalitis, California encephalitis, Sindbis virus infection, Tahyna virus infection, Congo-Crimean Hemorrhagic Fever (CCHF), sandfly viruses, hanta- and enterovirus. We are testing you to determine if you have an active infection with these bacteria or viruses.

Purposes of this Study:

1. To see how many hospitalized people in Kazakhstan have antibodies to the infections mentioned above.

2. To figure out risk factors associated with prior exposure to these infections

3. To aid in the development of future scientific research and developing preventative programs and treatment of disease

Eligibility for Participation:

To join this study, you have a onging fever where the reason is not known for.

You cannot join this research study if you are less than 15 years of age.

Screening Procedures:

If you agree to participate in this study, one of the study team members will ask about your understanding of the study and if you have any questions. They will make sure you are eligible to participate in the study.

Collection of Samples:

We will draw approximately 20 ml (approximately four teaspoons) of blood from a vein in your arm twice: in the day of hospitalization and on 7-14 day of disease. We will also ask you some questions about the possibility that you have had a prior exposure to the infections to be studied. You will not have to do anything other than give blood and answer questions. As a volunteer you do not have to answer any question(s) about which you feel uncomfortable.

Specimen Testing:

Your blood will be tested for antibodies against the infections we mentioned earlier in the consent form. Testing could occur at the Bundeswehr Institute of Microbiology (InstMikroBioBw, Germany), at the SPC SEEM, in Kazakhstan, at the KazNMU (Kazakhstan) or even laboratories at other places in the world (see future testing section). T

Test Results:

We will not inform you of the results of the testing. It is important that you understand that a positive result may mean you had one of these infections in the past. It does not mean that you are infected now. Therefore, the results of these tests do not affect your current health status.

Future testing:

We would like to save some of your blood sample for future tests possibly at Kazakhstan and German laboratories, or possibly other laboratories in other countries. At the end of this form, you will have the opportunity to decide whether or not to allow us to do that. Because medical technology is changing rapidly, we cannot tell you with confidence what types of studies we might conduct. However, any future studies will be limited to research broadly related to the present research objectives. Any sample of your blood used for future testing will be labelled only with a study number and will not be linked to any of your personal information. You will not receive a report on any results that may come from future studies.

Duration of Study:

Your participation in this study will take approximately 20 minutes. If you agree to future testing, we will retain your blood sample at the SPC SEEM, the KazNMU and the InstMikroBioBw, for 10 years. If you do not agree to future testing, we will dispose of your blood sample at the end of the study. We will retain papers from the study and the data at the SPC SEEM, the KazNMU and the InstMikroBioBw for 10 years.

Foreseeable Risks or Discomforts:

There are several small risks to participating in this study. There could be some discomfort associated with the needle stick for a blood draw. You could have swelling or bruising, and there is a small risk of infection at the site of the needle stick. Although most people have no infection or noticeable swelling, it cannot be prevented in all cases. In the majority of cases, such bruises will go away by themselves in 1 to 2 weeks. A few people feel light-headed and may develop a fast heartbeat during blood collection. These symptoms can be halted by having you lay down and/or by stopping the procedure. Rarely, you may develop a blood clot at the site of the blood draw. The area around this blood clot can become red and painful. The bump associated with the blood clot can persist for many weeks. These are the risks of obtaining blood samples, but significant complications do not often occur.

There is a small risk that your confidentiality may be breached. Measures to protect your personal information will be described in detail.

Benefit to Subject:

There is no direct medical/health benefit to you by participating in this study. However, this study may benefit the health of people in your community in the future.

Circumstances of Withdrawal:

Your participation is voluntary. You may withdraw from this study at any time without losing any benefits that you would otherwise have. Your participation may also be terminated without your consent if health conditions or other conditions occur that might be dangerous or detrimental to your health, you fail to comply with the procedures outlined in this informed consent, or the Sponsor terminates this study.

Confidentiality of Volunteers:

All data and medical information obtained about you, as an individual, will be considered privileged and held in confidence. You will not be identified by name in any published report or presentation of the results. The Kazakhstan authorities will receive a report containing grouped results, but will not be able to identify you individually. As part of their responsibility to oversee research and ensure protection of volunteers, the Kazakhstan authorities may inspect the records of this research. Regulatory groups in Kazakhstan and German Science Institutions may also inspect the records of this research. By signing this consent form, you agree to such inspection and disclosure. Complete confidentiality cannot be promised to volunteers because reporting information to appropriate medical or command authorities about your health may be required. Kazakhstan law requires us to ask for your permission to use your information for research. You can stop us from using your information at any time by contacting us

and asking us to stop. Signing the informed consent form you agree to participate in study.

CONSENT for the Use of Your Samples for Future Studies:

There is a possibility that the blood sample you are donating during this study may be used in other research studies and for other types of research tests. These tests may be performed possibly at the SPC SEEM, the KazNMU in Kazakhstan and the InstMikroBioBw in Germany, or possibly other laboratories in other countries. You will not be notified of future uses of your sample. Please indicate your willingness to permit this use of your donated sample by signing the appropriate statement:

Sample can be stored for future use for up to 10 years

Sample may be used in other research studies in the future

_____

Sample has to be used in the current study **only**.

Sample **cannot** be stored for future use. _____

CONSENT for Participation in the Research Study:

Your signature below indicates that you have read this informed consent document, the research study has been explained to you and your questions have been answered, and you agree to take part in this study. You will receive a copy of this signed form.

_____

Printed Name of Volunteer

_____

Permanent Address of Volunteer

_____

Printed Name of Person Conducting the Informed Consent Discussion

_____ _____

Signature of Person Conducting the Informed Consent Discussion

Date (dd/mm/yyyy)

_____

Printed Name of Witness (if applicable)

_____ _____

Signature of Witness (if applicable) Date (dd/mm/yyyy)

Informed Consent Document for parents of children (15 years and older) participating in

"Study of Patients with Fever of unknown origin due to selected Infectious Diseases in five regions (oblasts) of Kazakhstan"

Children with acute and current fever may have an acute infectious disease with certain bacteria and viruses. For these reasons, we want to test the blood of your child. We can with this test see if your child has been exposed to these infections, either recently or long ago. After a person is exposed to a virus or bacteria, they produce substances called antibodies. Antibodies are proteins made by the body's natural defense system (immune system) to attack and destroy foreign substances, such as bacteria and viruses. These antibodies can remain in the blood of people for many years after exposure to the infection. This study is designed to measure antibodies against some infections in children between 15 and 18 years.

The infections to be studied include brucellosis, leptospirosis, leishmaniosis, meloidosis, glanders, borreliosis, erlichiosis, anaplasmosis, Q-fever, spotted fever, plague, anthrax, tularaemia,typhus group and scrub typhus group rickettsioses, tick-borne encephalitis (TBE), West Nile fever, Dengue virus infections, Japanese encephalitis, California encephalitis, Sindbis virus infection, Tahyna virus infection, Congo-Crimean Hemorrhagic Fever (CCHF), sandfly viruses, hanta- and enterovirus. We are testing your child to determine if it has an active infection with these bacteria or viruses.

Purposes of this Study:

1. To see how many hospitalized children in Kazakhstan have antibodies to the infections mentioned above.
2. To figure out risk factors associated with prior exposure to these infections
3. To aid in the development of future scientific research and developing preventative programs and treatment of disease

Eligibility for Participation:

To join this study, your child should have an ongoing fever where the reason is not known for.

As rule, a child is legally unable to provide informed consent. For this reason before testing your child (<15 years) for mentioned above infections You (Parent(s)/legal guardian) have to sign an Informed Signed Form. You have the right to refuse to sign

this form without any justification nor prejudice in regard to services provided to him by the hospital. According to Kazakh ethics rules and Good Clinical Practice for the protection of people rights, a child cannot be included in this project without the parent's or his legal guardian's signature. Copies of the Informed Consent Form together with this Information Notice will be handed to you for your record if you accept that your child participates in this project.

Screening Procedures:

If you agree that your child participates in this study, one of the study team members will ask about your understanding of the study. If you have any questions, they will make sure that your child is eligible to participate in the study.

Collection of Samples:

We will draw approximately 20 ml (approximately four teaspoons) of blood from a vein in the arm of your child twice: in the day of hospitalization and on 7-14 day of disease. We will also ask you and your child some questions about the possibility that you have had a prior exposure to the infections to be studied. Your child will not have to do anything other than give blood and with your help to the answer questions. As a volunteer your child does not have to answer any question(s) about you or your child feel uncomfortable.

Specimen Testing:

The blood sample of your child will be tested for antibodies against the infections we mentioned earlier in the consent form. Testing could occur at the Bundeswehr Institute of Microbiology (InstMikroBioBw, Germany), at the SPC SEEM, in Kazakhstan, at the KazNMU (Kazakhstan) or even laboratories at other places in the world (see future testing section).

Test Results:

We will not inform you and your child of the results of the testing. It is important that you understand that a positive result may mean your child had one of these infections in the past. It does not mean that your child is infected now. Therefore, the results of these tests do not affect the current health status of your child.

Future testing:

We would like to save some of your child blood sample for future tests possibly at Kazakhstan and German laboratories, or possibly other laboratories in other countries. At the end of this form, you will have the opportunity to decide whether or not to allow us to do that. Because medical technology is changing rapidly, we cannot tell you with confidence what types of studies we might conduct. However, any future studies will be

limited to research broadly related to the present research objectives. Any sample of your blood used for future testing will be labelled only with a study number and will not be linked to any of your personal information. You and your child will not receive a report on any results that may come from future studies.

Duration of Study:

The participation of your child in this study will take approximately 20 minutes. If you agree to future testing, we will retain blood sample of your child at the SPC SEEM, the KazNMU and the InstMikroBioBw, for 10 years. If you do not agree to future testing, we will dispose of this blood sample at the end of the study. We will retain papers from the study and the data at the SPC SEEM, the KazNMU and the InstMikroBioBw for 10 years.

Foreseeable Risks or Discomforts:

There are several small risks to participating in this study. There could be some discomfort associated with the needle stick for a blood draw. Your child could have swelling or bruising, and there is a small risk of infection at the site of the needle stick. Although most people have no infection or noticeable swelling, it cannot be prevented in all cases. In the majority of cases, such bruises will go away by themselves in 1 to 2 weeks. A few people feel light-headed and may develop a fast heartbeat during blood collection. These symptoms can be halted by having you lay down and/or by stopping the procedure. Rarely, your child may develop a blood clot at the site of the blood draw. The area around this blood clot can become red and painful. The bump associated with the blood clot can persist for many weeks. These are the risks of obtaining blood samples, but significant complications do not often occur.

There is a small risk that your child confidentiality may be breached. Measures to protect his/her personal information will be described in detail.

Benefit to Subject:

There is no direct medical/health benefit to your child by participating in this study. However, this study may benefit the health of people in your community in the future

Circumstances of Withdrawal:

The participation of your child in this study research is voluntary. Your child may withdraw from this study at any time without losing any benefits that you would otherwise have. The participation of your child may also be terminated without your consent if your child health conditions or other conditions occur that might be dangerous or detrimental to his/her health, you or your child fail to comply with the procedures outlined in this informed consent, or the Sponsor terminates this study.

Confidentiality of Volunteers:

All data and medical information obtained about your child, as an individual, will be considered privileged and held in confidence. Your child will not be identified by name in any published report or presentation of the results. The Kazakhstan authorities will receive a report containing grouped results, but will not be able to identify your child individually. As part of their responsibility to oversee research and ensure protection of volunteers, the Kazakhstan authorities may inspect the records of this research. Regulatory groups in Kazakhstan and German Science Institutions may also inspect the records of this research. By signing this consent form, you agree to such inspection and disclosure. Complete confidentiality cannot be promised to volunteers because reporting information to appropriate medical or command authorities about your child health may be required. Kazakhstan law requires us to ask for your permission to use your information for research. You can stop us from using your child information at any time by contacting us and asking us to stop. Signing the informed consent form you give the permission and agreement to your child to participate in study.

CONSENT for the Use of your child blood samples for Future Studies:

There is a possibility that the blood sample your child is donating during this study may be used in other research studies and for other types of research tests. These tests may be performed possibly at the SPC SEEM, the KazNMU in Kazakhstan and the InstMikroBioBw in Germany, or possibly other laboratories in other countries. You and your child will not be notified of future uses of your sample. Please indicate your willingness to permit this use of your child donated sample by signing the appropriate statement:

Sample can be stored for future use for up to 10 years

Sample may be used in other research studies in the future

_____

Sample has to be used in the current study only.

Sample cannot be stored for future use. _____

CONSENT for Participation in the Research Study:

Your signature below indicates that you have read this informed consent document, the research study has been explained to you and your questions have been answered,

and you give the permission to your child to take part in this study. You and your child will receive a copy of this signed form.

_____

Printed Name of Volunteer (a pediatric subject)

_____

Printed Name of parent's(s)/ a legal guardian

_____

Permanent Address of Volunteer (a pediatric subject)

_____

Printed Name of Person Conducting the
Informed Consent Discussion

_____ _____

Signature of Person Conducting the Informed Consent Discussion | Date (dd/mm/yyyy)

_____

Printed Name of Witness (if applicable)

_____ _____

Signature of Witness (if applicable) | Date (dd/mm/yyyy)

Annex 7 Questionnaire

General Information – The following questions should be filled by the interviewer			
1.1	**MODULE 1** **SOCIODEMOGRAPHICS**		
1.2	Hospital ID number		|__|__|__|
1.3	Date of Interview *(DD.MM.YYYY)*		|__|__| . |__|__| . |__|__|__|__| Day Month Year
1.4	Participant ID		|__|__|__|__|__|__|__|
1.5	Sex of participant	☐1 ☐2	Male Female
1.6	Name of Study Site	☐1 ☐2 ☐3 ☐4 ☐5	Almaty region East Kazakhstan North Kazakhstan West Kazakhstan Kyzylorda
Start of Interview – The following questions should be asked to the patient			
1.7	When were you born? *(INT: Enter "99" if day and/or month is unknown and 9999 if year is unknown)*		|__|__| . |__|__| . |__|__|__|__| Day Month Year
1.8	How old are you now? *(Enter "999" for declined to answer)*		|__|__|__| Age in years
1.9	What is your present marital status?	☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7	Single married Not married, living with permanent partner Separated/divorced Widowed Declined to answer Other (*please specify*) _____
1.10	Where were you born? *(country of birth)*	☐1 ☐2	Kazakhstan Kirgistan

		☐3	Usbekistan
		☐4	Other country *(please specify)*: _____
1.11	In which oblast of Kazakhstan were you born?		_____
1.12	In which City/Town/Village do you live?		_____
1.13	Since when have you been living in this City/Town/Village? *(Enter "999" for declined to answer/don't know)*	☐1 ☐2	Always lived in this place Since |__|__|__| Years
1.14	Have you done any trips from your place of residence within the last month?	☐1 ☐2	No Yes (*please specify where*): _____
1.15	Have you done a trip into nature?	☐1 ☐2	No Yes (*please specify where, when and how often*): _____ _____ _____ _____
1.16	Have you had contact with wild animals?	☐1 ☐2	No Yes (*please specify*): _____
1.17	Have you been biten by ticks, mosquitoes, insects or wild animals within the last month?	☐1 ☐2	No Yes (*please specify*) _____

MODULE 2 EDUCATION AND WORK

2.1	What is your highest level of education?	☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ 9 ☐ 10 ☐ 11	Still in school (pupil/student) Still in college (student) Still in academy/institute/university (student) Secondary education Secondary education unfinished Any vocational education Vocational education unfinished Any higher education Higher education unfinished Have no formal education Declined to answer/Don't know	
2.2	What is your current occupation?	Full -time	Part-time	
	(Multiple answers allowed. For each single answer please ask If the work is done full-time or part-time)	☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐8 ☐9 ☐ 10	Pupil/Student (full-time) Farmer/Peasant/Farmworker (plants) Farmer/Peasant/Farmworker (animal) Farmer/Peasant/Farmworker (forestry) Keeping the house (housewife) Unskilled Labourer [1] _____ Skilled Labourer [2] _____ Local or long distance driver Administrative or academic professional [3] Businessman/woman Nurse/Physician/Clinician/Pharmacist

		☐ 6 ☐ 7 ☐ 8 ☐ 9 ☐ 10 ☐ 11 ☐ 12 ☐ 13 ☐ 14	☐ 11 ☐ 12 ☐ 13 ☐ 14	Unemployed Declined to answer Other (please specify) _____
		[1] *please specify* [2] *factory worker, mechanic, painter, welder, carpenter, dressmaker, technician…* [3] *secretary, bank clerk, teacher, pastor, surveyor, lawyer, accountant, engineer, labworker, scientist…*		
2.3	How long have you been working in your current occupation? *(Enter "99" for declined to answer/don't know)*		|__|__| Years	
2.4	How often do you normally work in the gardens and fields? *(plant cultivation)*	☐1 ☐2 ☐3 ☐45	Yes, always/daily Yes, often Yes, occasionally No, never	
2.5	Can you read a letter or newspaper easily,	☐1 ☐2	Easily With difficulty	

	with difficulty, or not at all?	☐3 ☐4	Not at all Declined to answer
2.6	What is the total cash income of your household per year?	☐1 ☐2 ☐3 ☐4	Low (below 900,000 tenge, 5,000 $ per year) Medium (900,000 – 1,800,000 tenge, 5,000 - 10,000 $ per year) High (> 1,800,000 tenge (>10,000 $ per year) Decline to answer/do not know

Module 3 LIVING AND HOUSING				
3.1	How many people do normally eat together in your household? *(Enter "99" for declined to answer/don't know)*		|__|__| Number	
3.2	What type is your flat/house?	☐1 ☐2 ☐3 ☐4	Well-equipped city apartment Poorly equipped city apartment Private well-equipped house Poorly equipped house Other *(please specify):* _____	
3.3	Where do you store your bulk products (flour, sugar, rice, etc…)?	☐1 ☐2 ☐3	In bags In casks Other (*please specify*) _____	
3.4	From where do you get your water?	☐3 ☐4 ☐5 ☐5	city water pipe rural water pipe blow well river	
3.5	Did you drink raw milk directly from the animal or eat raw milk	☐1 ☐2	No Yes If yes, from which animal?	

	products without pasteurization?		_____	

MODULE 4: livestock			
4.1	What kind of livestock does your household own and how many of each species? (*if the real number is unknown, please estimate – Enter "000" for animal if none and "999" for don't know*)		Cattle |__|__|__| Number Horse |__|__|__| Number Goats |__|__|__| Number Sheep |__|__|__| Number Pigs |__|__|__| Number Poultry |__|__|__| Number Cats/Dogs |__|__|__| Number other: _____
4.2	Did the animals have any kind of disease?	☐ 1	Yes If yes, which kind of animal? _____
		☐ 2	No
4.3	Which symptoms did you notice?	☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5	Unusual movement Respiratory symptoms Gastroenterological symptoms Lesions Others, please specify: _____ Don't know

		☐ 6				
4.4	Did you notice an unusual high number of animal deaths?	☐ 1	No			
		☐ 2	Yes If yes, which animal? _____			
4.5	Do you have contact with died animals?	☐ 1 ☐ 2	No Yes If yes, which animal? _____			
4.6	How often do you have direct contact to the following animals? *("Contact" means working with these animals, milking, slaughtering, handling raw meat), but* ***NOT*** *eating cooked meat*		Always/daily	Most of the times	Rarely	Never
	Cattle?		☐1	☐2	☐3	☐4
	Horse		☐1	☐2	☐3	☐4
	Goats?		☐1	☐2	☐3	☐4
	Sheep		☐1	☐2	☐3	☐4
	Pigs?		☐1	☐2	☐3	☐4
	Cats/Dogs		☐1	☐2	☐3	☐4
	Poultry?		☐1	☐2	☐3	☐4
4.7	Do you handle raw meat? *(slaughtering, butchering, preparing for cooking)*	☐ 1 ☐ 2 ☐ 3 ☐ 4	Yes, always Yes, most of the times Yes, but rarely No, never			

4.8	How often do you see/notice rats or mice (or bat poop)	☐ 1 ☐ 2 ☐ 3 ☐ 4	Always Most of the times Rarely Never
4.9	How often do you have to kill them in the house?	☐ 1 ☐ 2 ☐ 3 ☐ 4	Always Most of the times Rarely Never
4.10	Do you have bird nests in the roof?	☐ 1 ☐ 2 ☐ 3	Yes No Don’t know
4.11	Do bats live in your house or in trees around your house?	☐ 1 ☐ 2 ☐ 3	Yes No Don’t know

MODULE 5: VECTOR HABITAT FACTORS			
5.1	Where is your house located, in urban or rural area?	☐1 ☐2	Rural area Urban area
5.2	What kind of ground is around your residence?	☐1 ☐2 ☐3	Tarmac Sand Dirt
5.3	How does the vegetation around your residence look like? *(Multiple answers allowed – mark all that apply.)*	☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7	Dense plantation/forest Larger grass fields Occasional bush agricultural fields Swamp Lake Forest Others *(please specify)*:
5.4	Is there sometimes **standing** water close to your residence? *(Multiple answers allowed)*	☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐7	Yes: Puddles after heavy rain Containers for collecting water Lake stream Gully in urban area No, never Don't know
5.5	How many months per year is that water around your residence?		|__|__| Number of month

MODULE 6: CLINICAL SYMPTOMS			
6.1	Beginning of the symptoms	☐1 ☐2	> 5 days ago < 5 days ago

6.2	Please indicate below which symptom(s) you have:	☐1 ☐2 ☐3 ☐4 ☐5 ☐6 ☐7 ☐8 ☐9 ☐ 10 ☐ 11 ☐ 12 ☐ 13 ☐ 14 ☐ 15 ☐ 16 ☐ 17 ☐ 18 ☐ 19	Fever Headache Neck pain (meningism) Weakness of muscels or joints Muscle pain or recurrent cramps Pain on swallowing Joint pain Stomach/abdominal pain/cramps Back pain Earache Cough Difficulties in speaking, hearing or seeing Seizures/Epilepsy Difficulties in breathing, Rapid breathing Sore threat Congestion of nose Enlarged lymph nodes Icterus
6.3	Body temperature (*ear measurement*)	☐1 ☐2	< 37.5°C > 37.5°C *If body temperature is > 37.5°C, than fill out 6.4*
6.4	Duration of high body temperature	☐1 ☐2	< 3 days 3 days and more
6.5	Blood pressure	☐1 ☐2	Normal Hypotension

		☐3	Hypertension
6.6	Pulse rate	☐1 ☐2 ☐3	< 80 80 – 100 > 100
6.7	Skin conditions	☐1 ☐2 ☐3 ☐4	Exanthema Ulceration Edema Others (*please specify*) _____
6.8	Stool	☐1 ☐2 ☐3 ☐4	Diarrhoea Blood in stool Bright stool Others (*please specify*) _____
6.9	Urine	☐1 ☐2 ☐3 ☐4 ☐5	Blood in urine Pain on urinating Dark urine Low urine volume Others (*please specify*) _____
6.10	Medications	☐1 ☐2 ☐3 ☐4	Antipyretics Antirheumatics Antibiotics Others (*please specify*) _____
6.11	Duration of therapy		__ Days
6.12	Similar illnesses in the family or in the surrounding	☐1 ☐2	Yes No