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Investigations on tick-borne bacterial agents in Kazakhstan

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Nurkeldi Turebekov

born in

Aktobe city, Republic of Kazakhstan

submitted on

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Supervisors LMU:	Title, first name, last name
Habilitated Supervisor	Prof. Michael Hoelscher
Direct Supervisor	PD Dr. Sandra Essbauer
3rd LMU Supervisor	Dr. Guenter Froeschl

Supervisor External:

Local Supervisor	PD Dr. Gerhard Dobler

Reviewing Experts:

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

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

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Abstract

Background During the past 23 years the incidence of tickborne rickettsioses increased in Kazakhstan but studies on epidemiological data, vector species, prevalence and distribution are still insufficient to date. Unfortunately most cases of rickettsioses are remained unconfirmed due to the lack of modern diagnostic tests. The purpose of the research was molecular investigation of ticks for spotted-fever group rickettsiae in two pilot regions of Kazakhstan and to detect its role as a cause of the fever of unknown origin (FUO).

Methods Six different tick species were collected and sorted from two selected regions in Kazakhstan. DNA was isolated and all tick samples were investigated for the presence of Rickettsia by real-time PCR. Multi-locus sequence typing (MLST) was conducted for positive samples. Serological study on the presence of antibodies against Rickettsia was performed at the same regions with serum samples from 802 patients with FUO. Statistical analyses were done with R.

Results Rickettsial minimum infection rate in collected ticks varied within 0.4–15.1% in Almaty region and 12.6–22.7% in Kyzylorda region. Four different *Rickettsia* species were identified. Two of them are already known: *Rickettsia raoultii* and *R. slovaca*, the latter was detected for the first time in Almaty region. And two new – "*Candidatus* R. yenbekshikazakhensis" and "genotype R.talgarensis" – were identified by MLST. In the serological study, 11 (1.4%) patients with acute tick-borne rickettsiosis and 22 (2.7%) patients with acute typhus rickettsiosis were detected.

Conclusion Kazakh clinicians should be aware of *Rickettsia* spp. circulating in both investigated regions. *R. raoultii* and *R. slovaca,* which are human pathogenic, should be included in the diagnostics. The human pathogenicity of the two newly described rickettsiae has to be further investigated. Further rickettsioses can be a cause of FUO, which has to be taken into account for diagnostics.

Declaration of Published Contents

Parts of this thesis have been published in the peer-reviewed paper

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List of Abbreviations

CFT	Complement fixation test
CI	Confidence interval
DEBONEL	*Dermacentor*-borne necrosis erythema and lymphadenopathy
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ELISA	Enzyme-linked immunosorbent assay
FUO	Fever of unknown origin
*glt*A	Citrate synthase gene
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MIR	Minimum infection rate
MLST	Multi-locus sequence typing
*omp*AIV	Outer membrane protein A IV
*omp*B	Outer membrane protein B
OR	Odds Ratio
OR a	Adjusted Odds Ratio
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rt-PCR	Real time polymerase chain reaction
*sca*4	Cell surface antigen 4 gene
SENLAT	Scalp eschar with neck lymphadenopathy
SFG	Spotted fever group
SOP	Standard operating procedure
TG	Typhus group
TIBOLA	Tick-borne lymphadenopathy
UDG	Uracil-DNA-glycosylase

1. Introduction

1.1. Overview on the spotted fever group of Rickettsia

This research work has been devoted to the investigation of rickettsial bacterial agent that causes various types of spotted fevers and typhoid fever around the world (1, 2).

Rickettsia are small (0.3 µm – 1.0 µm), aerobic, motionless, non-spore formed, gram-negative, arthropod-associated microorganism with obligate intracellular life cycles in eukaryotic cells, such as endothelial cells. Rickettsia species are pleomorphic bacterial agents, which can mostly form cocci and bacilli. This microorganism has the characters of bacteria and viruses at the same time. As a bacterium, Rickettsia has own cell membrane, both nucleic acids (DNA and RNA) and multiply by binary fission in the cytosol. In comparison to viruses, Rickettsia are intracellular parasite with several species causing cell death and can be cultivated only by using cell cultures or eggs cultures (3).

Historically, this rickettsiae were discovered more than 100 years ago, by the American pathologist and microbiologist Howard Taylor Ricketts (1871 – 1910). He dedicated the last decade of his life for investigation of the causative agents and mode of transmission of Rocky Mountain spotted fever (USA) and epidemic typhus in Mexico City (Mexico), where he died. He discovered that Rickettsiae are arthropod-borne microorganisms, which can be found in ticks, lice, fleas. Later, in memory of H.T. Ricketts, the genus Rickettsia was named behalf his name (4).

During the past three decades, due to the implementation of the molecular biological methods and new identification techniques, such as multi-locus sequence typing (MLST) or next generation sequencing, the Rickettsia taxonomy was revised (5,6). For details of the general taxonomy of rickettsia see figure 1.1 on the next page.
At the present time, the genus *Rickettsia* is represented with 32 officially recognized and validated species, which are spread all over the world, excluding Antarctica. Almost half of them are identified and confirmed as human pathogens, which can cause various diseases from subclinical to severe forms. The pathogenicity of others rickettsial agents so far is not yet detected. As arthropod-borne bacterial organisms, *Rickettsia* species are mostly transmitted by Ixodid ticks, lice, fleas or mites, which can be not only the vectors but also their reservoirs for Rickettsia (2).

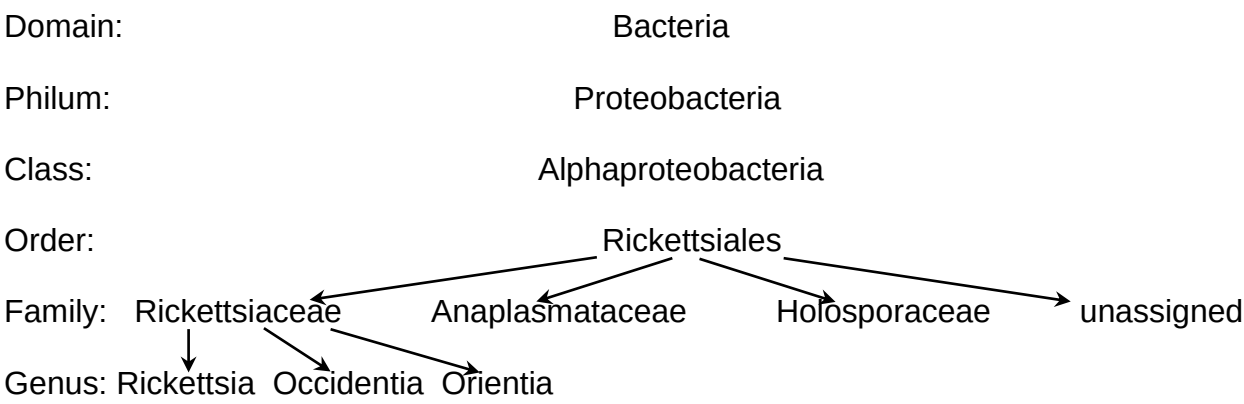

Figure 1.1 Taxonomy of Rickettsia.

According to the newest scientific classification (2018), all *Rickettsia* spp. are divided into two major groups: spotted fever group of Rickettsia and typhus group of Rickettsia (Table 1.1) (7).

Table 1.1 Modern classification of Rickettsia.

Spotted fever group of Rickettsia	Typhus group of Rickettsia
Spotted fever group	Typhus group
R. rickettsia subgroup	"Ancestral" group
R. conorii subgroup	*R. bellii* subgroup
R. australis subgroup	*R. canadensis* subgroup
R. felis group	
R. akari group	

During the last decades, the knowledge for the spotted group of rickettsiae and its significance of inducing human diseases has been considerably enhanced. The new innovation molecular techniques, such as multi-locus sequence typing lead to the description of several new *Rickettsia* Candidatus species by describing at least four or five gene fragments or new *Rickettsia* genotypes if less than four sequences are characterized (2, 8-10).

1.1.1 Spotted fever group of Rickettsia in Kazakhstan

In Kazakhstan the clinical picture of human cases of tick-born rickettsiosis were first described during expeditions to Almaty region already in 1949 to 1951 (11). A few years later clinical pictures of tick-borne rickettsiosis were described further in five districts i.e. South Kazakhstan, West Kazakhstan, Pavlodar, North Kazakhstan and Akmola regions (12). The causative agent of North Asian tick-borne rickettsiosis (*R. sibirica*) was first described and isolated in 1961 by intra-abdominal infection of guinea pig males with homogenates containing *Dermacentor marginatus* and *Haemaphysalis punctata* ticks, which were collected in Yenbekshikazakh district of Almaty region (13).

Since only 1995 there exits registration of tick-borne rickettsioses cases in humans. According this a clinical case definition criterion and a complement fixation test (CFT) with *R. sibirica* are used in Kazakhstan for diagnostics and thus for registration. So far for four regions in Kazakhstan four regions are currently considered as endemic regions for tick-borne rickettsioses, which are North Kazakhstan, Pavlodar, East Kazakhstan and Kyzylorda (Fig. 1.2).

So, case numbers are registered in these four regions. According to these available reports, in total 3.904 human cases of tick-borne rickettsiosis were officially registered in Kazakhstan from 1995 to 2016. During this time the incidence rate of this disease rise from 0.41 to 1.19 (per 100,000 inhabitants per year). The highest increase was found during this period in Kyzylorda (1.64 – 11.1 per 100,000 inhabitants per year) and Pavlodar (1.07 – 7.0 per 100,000 inhabitants per year) regions. Annual development of incidence is shown in Figure 1.3. With the present available data, the Kyzylorda region is presumed to be the most endemic area for tick-borne rickettsioses in Kazakhstan (14).

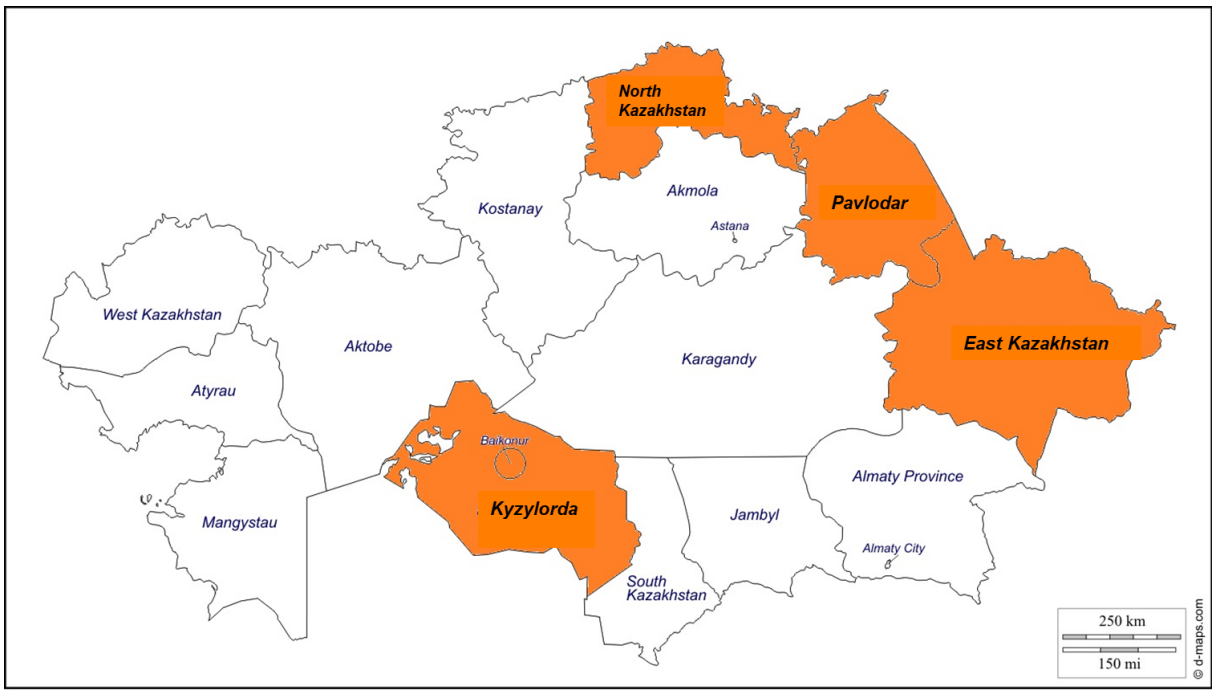

Figure 1.2 Localization of tick-borne rickettsioses in Kazakhstan.

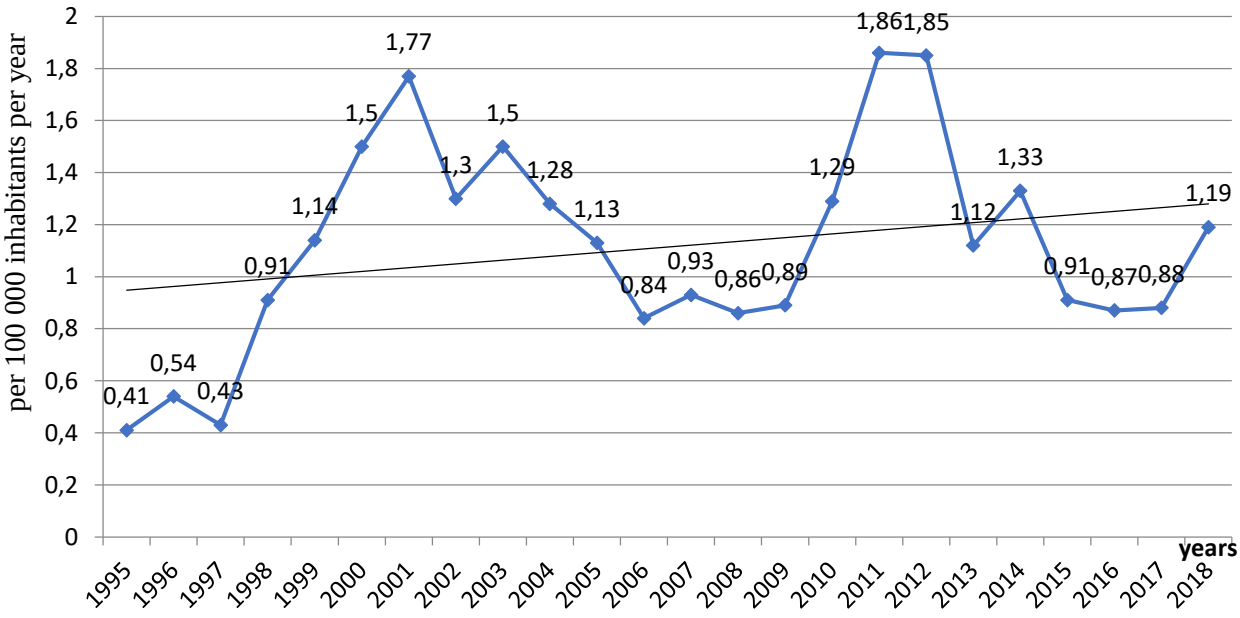

Figure 1.3 Incidence of tick-borne rickettsiosis in Kazakhstan based on complement fixation test. Source: Annual report: Epidemiological situation of infectious diseases in the Republic of Kazakhstan for 2018 (14).

So far, we can only find limited data on *Rickettsia* species that might be present in the endemic regions of Kazakhstan. *R. raoultii* was found in *Dermacentor* spp. and *Ixodes* spp. in three regions Kyzylorda, Karaganda, and East Kazakhstan, Kazakhstan

(15 –20). Further, *R. conorii* subsp. *caspia, R. raoultii* and *R. aeschlimannii* were described in ticks originating from Western, Northern and Central regions of Kazakhstan (21). *R. aeschlimannii* was detected in *Haemaphysalis punctata* in Almaty region (17, 18). Recently, American colleagues described *R.conorii* subsp. *caspia* in ticks taking a blood meal on four-striped grass rats (*Rhabdomys pumilio*) originating from the West Kazakhstan region. In 2017 *Rickettsia asembonensis* and *Rickettsia felis/Candidatus* Rickettsia senegalensi were described in fleas sampled in Almaty region (12, 22, 23).

All these hints from references show that there is a quite limited knowledge on *Rickettsia* spp. from the spotted fever group circulating in the Almaty region which is the most densely populated region in Kazakhstan. Despite some old data Almaty region is not named as an endemic region and therefore there are no registered epidemiological data on human infections from Almaty region (24). Therefore, presently there are still big gaps regarding the knowledge on circulating *Rickettsia* species in ticks and their geographical distribution in Kazakhstan. This research work present data of a study of molecular investigation of ticks for SFG of Rickettsia in the Almaty region, which is considered so far non-endemic, and in the endemic Kyzylorda region.

1.2 Rickettsia as a causative agent of the fever of unknown origin

Despite the advances achieved in diagnosis and treatment in recent decades, fever of unknown origin (FUO) still remains a challenging clinical problem all over the world, especially in developing countries. For the first time, the concept of fever of unknown origin was defined by R.G. Petersdorf and P.B. Beeson in 1961 which has not changed over the next 30 years (25). After this time, in 1991 it was suggested to classify FUO into four types (classical, nosocomial, neutropenic and HIV-associated FUO) and proposed to reduce the duration of the human diagnostic evaluation from one week to three outpatient visits or three days inpatient examination (26).

To date, there are more than 200 diseases that are accompanied by fever of unknown origin (27). In Kazakhstan, so far, no study on patients with fever of unknown origin was conducted. Herein, I have focused on investigating antibodies against the group of the wide spread intracellular bacteriae from the genus *Rickettsia* in patients having FUO.

Rickettsia of the typhus group are *R. prowazekii*, which is transmitted by the human body louse, and is a causative agent of epidemic typhus (louse-borne typhus). Outbreaks of louse-borne typhus often occur among the representatives of the poor strata of the population during the cold seasons (28). According to WHO, the recent outbreaks of typhus fever have been reported in central and eastern Africa (Burundi, Ethiopia, Rwanda), central and South America, and Asia (29). Further, *R. typhi* is a member of the typhus group (TGR, typhus group of Rickettsia) transmitted by fleas and causes murine typhus (endemic typhus, flea-borne typhus). Mostly, the persons who come into contact with flea-infested domestic and wild animals (rodents) are at high risk for endemic typhus (28). Flea-borne typhus is widely distributed around the world, mainly in rat-infected tropical and subtropical areas (30).

Rickettsiae of the spotted fever group (SFG, spotted fever group) are pathogens which induce a number of tick-transmitted rickettsioses including Rocky Mountain spotted fever, Mediterranean spotted fever, Far Eastern spotted fever, North Asian tick typhus, Astrakhan spotted fever etc (28). Tick-borne rickettsioses are distinguished by its natural focality and endemicity, which is often reflected in the name of a rickettsiosis.

Many types of rickettsial diseases (louse-, fleas-, tick-bite) are characterized by similar general symptoms such as developing within 1 – 2 weeks of infection and including fever, headache, malaise, rash (maculopapular, vesicular, or petechial), nausea, and vomiting (28). In this regard, the clinical diagnosis of rickettsiosis presents certain difficulties for practitioners. Some tick-bite rickettsioses are characterized by certain typical signs that facilitate the diagnosis by clinicians in this group of patients (28-33). For to date, it is well-known that *R. slovaca* and *R. raoultii* are the causative agents of tick-borne lymphadenopathy (TIBOLA) syndrome which is also named as *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL) and common in Southern and Eastern Europe and Asia (28). The main symptoms of this disease are asthenia (in 70 – 100% cases), painful lymphadenopathies (in 69 – 100% cases), painful eschar (in 64 – 100% cases) and fever (in 54 – 80% cases) (31). In publications, this syndrome is also called as scalp eschar with neck lymphadenopathy (SENLAT) and recently, it became known that one more rickettsial pathogen (*R. massiliae*) is responsible for the onset of this disease (32, 33).

The data concerning the circulating tick-borne *Rickettsia* species in Kazakhstan were described above.

As for epidemic and murine typhus, there is no official registration and no reliable diagnostic tests available in Kazakhstan. Mostly, Kazakh practitioners diagnose typhus based on epidemiological data and typical patient clinical pattern.

Until now, so far in Kazakhstan no large-scale serological study for the prevalence of antibodies against rickettsia has been conducted.

2. Rationale and Objectives

Despite the existence of recognized endemic areas in Kazakhstan, the annual cases of tick-associated rickettsiosis are unknown especially in the non-endemic regions of the country. So far there is lack of data concerning on the prevalence of selected agent in vectors such as ticks in natural foci and on serological prevalence of antibodies against spotted fever group of Rickettsia in the Republic of Kazakhstan.

The above-mentioned challenges have led us to highlight the following research objectives:

1. Look at the prevalence of selected bacterial agent (Rickettsia) in ticks collected from two selected regions of Kazakhstan
2. Explore the serological prevalence of antibodies against tick-transmitted Rickettsia in two selected regions
3. Molecular genetic characterization of the circulating species of Rickettsia.

3. Methods

3.1. Tick sampling

Ticks were collected by flagging, i.e. drawing a 1 m^3 sheet along, the vegetation in three districts of Almaty region (Talgar, Yeskeldy and Yenbekshikazakh districts) and Kyzylorda region (Syrdarya, Shyeli and Zhanakorgan districts), Kazakhstan, in May/June 2015.

In Almaty region three sample sites were chosen and ticks were collected there: (i) Yeskeldy district (44°54′12″N, 78°29′42″E) with Tekeli city (44°49′48″N, 78°49′26″E) is located in Almaty region adjacent to the People's Republic of China and is characterized by coniferous forests and open steppe vegetation. The region is situated mountaineous at an altitude of 1400–2200 m above sea level (asl). More than 40% of the area is covered by forest, and the remaining parts constitute of pasture and agricultural land. Animal husbandry is practiced widely. The average annual precipitation has been reported as 250–300 mm (34). (ii)Talgar district (43°18′55″N, 77°14′35″E) with Talgar city (43°18′0″N, 77°14′0″E) is 40 km away from Almaty city center and comprises of forested taiga, forested steppe, and arid fields, the latter mainly covered by gramineous plants. Nearly 20% of its northern part is elevated at 1800 to 2400 m asl and is therefore mountaineous. The annual precipitation is 200–300 mm (34). (iii) Yenbekshikazakh district (43°21′0″N, 77°28′0″E) with Yesyk city (43°21′0″N, 77°28′0″E) offers areas of maritime climate in summer and very cold temperatures (from -25°C down to -50°C) during winter. Average precipitation is reported as 200–700 mm/year. Plain steppe and meadows dominate most parts of this area (34).

In the Kyzylorda region ticks were collected in three districts: Syrdarya (45°34′12″N, 65°36′0″E), Shyeli (44°10′0″N, 66°44′0″E) and Zhanakorgan (43°56′24″N, 67°13′12″E) districts. Kyzylorda region (45°0’0’’N, 64°0’0’’E) is located in the south-western part of Kazakhstan, to the east of the Aral Sea in the lower reaches of the Syrdarya river, mainly within the Turan Lowland (altitude 50-200 m above sea level). The region borders the neighboring country Uzbekistan, as well as three other Kazakh regions: Aktobe region (to the west), Karaganda region (to the north), and South Kazakhstan (to the east). The climate is rather continental and extremely arid with prolonged hot and dry summers and with a comparatively warm, short and little snowy winter. The amount of precipitation in the north-west near the Aral Sea coast is about 100 mm (the lowest in Kazakhstan), in the southeast in the foothills of

Karatau mountain is up to 175 mm. A significant part of the region is occupied by sands, almost devoid of vegetation (35).

3.2 Tick sample preparation and DNA isolation from ticks

Ticks, collected by flagging in the field, were brought to the laboratory and stored at −20°C until further investigation. The laboratory investigation was performed in batches. After thawing, all field ticks have been sorted according genus, species, stage and sex following the official guidelines for tick specification in Kazakhstan (36-39). Later the ticks were grouped into pools by genus, species, stage and sex (with a maximum of 5 imago ticks in a pool). Each pool has been homogenized using the "Tissue Lyser II" instrument machine, after adding ceramic granules and 1 ml medium "DMEM" (BioloT, Saint-Petersburg, Russia) to each tube. Following Kazakh guidelines for biosafety and biosecurity aliquots containing tick homogenates were inactivated in the water bath at 56°C for 30 min, before DNA extraction. DNA was extracted from 200 µl tick homogenates using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final elution volume was 50 µl DNA.

3.3 Screening PCR and rickettsial species identification by multi locus sequence typing (MLST)

All PCRs were conducted in a three-room-regime following the one-way-principle.

In a first step for all samples the presence of rickettsial DNA was determined by a real-time PCR assay targeting the pan-rickettsial citrate synthase gene (primers and probe see Table 3.1) using Uracil–DNA–glycosylase (UDG) in order to eliminate carry-over contamination (40, 41) in a "Rotor-Gene Q" (Qiagen) machine.

In all samples yielding a positive pan-rickettsial citrate synthase gene signal, a multi-locus sequence typing (MLST) targeting six gene fragments (partial fragments of *ompB, ompA*IV*, 23S-5S* interspacer*, 16S, sca4, gltA*) was conducted for Rickettsia species identification (40-43). Firstly, for all these samples the partial outer membrane protein B (*ompB*) gene (RR 120-2788, cRR 120-3599) was amplified following published protocols (43). Secondly, for the samples with positive sequences (depending on the sequence result) five additional fragments were investigated using previously published primers (42, 40–47): *ompA*IV (RR 190-5125, cRR 190-

6013), *23S-5S* interspacer (23s for, 23s rev), *16S* (Ric, Ric RT), *sca4* (Rsca4_1707f, Rsca4_2837r) and partial *gltA* (Rh314, Rh654) (Table 3.1) (40-43).

Table 3.1 summarizes an overview of all PCR primers used in the tests.

Table 3.1 Overview on used primers and PCRs

Fragment	Forward primer	Reverse primer	Length (bp)	Ref.
gltA*	PanRick_gltA_2_for: 5'-ATAGGACAACCGTTTATTT-3'	PanRick_gltA_2_rev: 5'-CAAACATCATATGCAGAAA-3'	90	41, 42
ompB	RR 120-2788: 5'-AAACAATAATCAAGGTACTGT-3'	cRR 120-3599 5'-TACTTCCGGTTACAGCAAAGT-3'	811	44
ompAIV	RR 190-5125: 5'-GCGGTTACTTTAGCCAAAGG-3'	cRR 190-6013: 5'-TCTTCTGCGTTGCATTACCG-3'	888	43
23S	Rick 23s for: 5´-gATAggTCgggTgTggAAgCAC-3'	Rick 23s rev: 5´-gggATgggATCgTgTgTTTCAC-3'	378 – 532	45, this study
16S	Ric: 5´-TCTAGAACGAACGCTATCGGTAT-3'	RicRt: 5'-TTTCATCGTTTAACGGCGTGGACT-3'	752	46
sca4	Rsca4_1707f: 5'-CTCTgAATTAAgCAATgCgg-3'	Rsca4_2837r: 5'-CCTgATACTACCCTTACATC-3'	1130	47
gltA	RH314: 5'-AAACAGGTTGCTCATCATTC-3'	RH654: 5'-AGAGCATTTTTTATTATTGG-3'	340	48

* real-time PCR, probe. PanRick_gltA_2_taq: 5'-6FAM-CCTGATAATTCGTTAGATTTTACCG-DB-3'

Ref. – reference, bp – base pairs

Master mix solution for *23S-5S* interspacer PCR (50 µl including 5 µl DNA) were prepared with 0.2 mM dNTP Mix (Thermofisher-Invitrogen, Schwerte, Germany), 0.5 µM of each primer (23s for, 23s rev), 1.5 U Platinum® *Taq* DNA Polymerase High Fidelity (Thermofisher-Invitrogen), 1x PCR buffer (Thermofisher) and 2.0 mM MgSO4 (Thermofisher). The initial denaturation was performed for 3 min at 95°C, 45 cycles of amplification each started with denaturation for 20 sec. at 95°C, annealing for 30 sec. at 57°C and elongation at 68°C for 60 sec. The amplification ended with a final elongation for 10 min at 68°C (44, 48).

Gene D (*sca4*) sequences were amplified with 0.2 mM dNTP Mix (Thermofisher- Invitrogen), 0.1µM of each primer (Rsca4_1707f, Rsca4_2837r), 1.0 U Platinum® Taq DNA Polymerase High Fidelity (Thermofisher-Invitrogen), 1x PCR buffer, 3 mM MgSO4 and 2µl DNA in a final volume of 50 µl for each reaction. After an initial denaturation for 3 min at 95°C, 40 cycles with denaturation for 30 sec. at 95°C, annealing for 35 sec at 53°C and elongation for 90 amplification ended with a final extension for 7 min at 68°C. The runs were finished with a final elongation for 7 min at 68°C (46, 48).

Partial *16S* sequences were amplified using 0.2 mM dNTP Mix (Thermofisher- Invitrogen), 0.5 µM of each primer (Ric, Ric RT) with 1.5 U Platinum® Taq DNA Polymerase High Fidelity (Thermofisher-Invitrogen), 1x PCR buffer, 2.5 mM MgSO4 and 5µl DNA in a final volume of 50 µl for each reaction. After an initial denaturation for 3 min at 95°C, 45 cycles with denaturation for 30 sec 94°C, annealing for 30 sec 63°C, and elongation for 120 sec at 68°C were performed. The PCRs were ended with a final extension for 7 min at 68°C (45, 48).

Finally, the partial *gltA* gene was amplified with 0.2 mM dNTP Mix (Thermofisher- Invitrogen), 0.5 µM of each primer (Rh314, Rh654), 1.0 Unit Platinum® Taq DNA Polymerase High Fidelity (Thermofisher-Invitrogen), 1x PCR buffer, 2 mM MgSO4 and 5 µl DNA in a final mixture of 50 µl for each reaction. After an initial denaturation for 2 min at 94°C, 45 cycles with denaturation for 20 sec at 94°C, annealing for 30 sec at 54°C and elongation for 60 sec at 68°C were conducted. Final extension was performed for 5 min at 68°C (47, 48).

PCR products were visualized in a 1.5% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's recommendations. PCR product sequencing was carried out using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3500xl Genetic Analyzer (Hitachi, Japan) with the same primers as those utilized for the initial PCR amplification. As a sequencing control was used pGEM -3Zf(+) control template. Quantification of the PCR products were performed on a Fluorometer Qubit 2.0 (Invitrogen, USA). Sequence analyses were done with Chromas Lite 2.01 (49) and Bioedit 7.2.5. (50). Obtained sequences were compared with sequences from GenBank using BLAST 2.2.32 (51, 52).

After alignment phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura 3-parameter model (53) in the software package MEGA (54). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The binomial (Clopper-Pearson) 'exact' method based on the beta distribution was used for the calculation of 95% confidence intervals.

The Minimum Infection Rate (MIR) was calculated as the ratio of the number of positive tick pools to the total number of ticks of the same species.

3.4 Description of variables in questionnaires

For each patient a questionnaire was filled out together with blood taking. The following variables were noted:

- 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°C, (2) More than 37.5°C.
- 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.

3.5 Serum sampling

For the serological investigation sera from patients with fever of unknown origin (FUO) were used. Paired sera (day 1, day 10-14) were collected from adolescence (>15 years old) and adult hospitalized patients with FUO during the warm season in 2015 – 2016 (April – October) from 13 hospitals in the Almaty (n=9) and Kyzylorda (n=4) regions of Kazakhstan. FUO was defined as fever more than 3 days with high temperature (>37.5 ºC ear temperature) and exclusion of rheumatic fever (autoimmune conditions). All enrolees completed a specially designed questionnaire with 56 questions concerning socio-demographic, living, livestock, vector habitat factors and clinical signs and signed informed consent forms at the time of sampling. Ethical issues were considered and endorsed by the national Kazakh Ethical Committees and in Germany by the ethical committee from the Ludwig-Maximilians University (Munich) respectively. For details on the questionnaire and informed consent forms see Appendix.

3.6 Serological investigations by Rickettsia typhi and Rickettsia spotted fever group ELISA

Collected blood was centrifuged and separated sera were frozen in four aliquots at -20°C. Sera samples were tested for the presence of immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies against TG (*R. typhi)* and IgG antibodies against SFG of Rickettsiae by using commercial enzyme-linked immunosorbent assay (ELISA)

kits (Fuller Company, USA) according to the standard operating procedures (SOPs) and manufacturer's guide.

During the first step, all collected second serum samples were screened for IgG (*R. typhi* and Rickettsia SFG) with appropriate ELISA kits. At the second step, the first and the second sera of all positive samples were retested and titrated for IgG (*R. typhi* and Rickettsia SFG) to identify acute and exposed infection by using conforming ELISA kits. In a third step, the first sera of all positive samples with supposed acute infection (*R. typhi*) were screened with IgM ELISA kits.

3.7 Statistical analysis

For statistical processing of the data derived from questionnaires, the R statistical environment, version 3.5.3 (55) with RStudio graphical front-end, version 1.1.463 (56) were used.

The influence of different demographical, environmental and occupational factors (predictors) on the positive results of tick-borne rickettsiosis or typhus group rickettsiosis (outcome variables) were tested in the binomial univariate regression model using the *glm* function of the R *stats* package.

At the next step, the odds ratios predicting the relationship between outcomes (tick-borne rickettsiosis or typhus group rickettsiosis) and factor variables were evaluated in the binomial multivariate model, in which factor variables were adjusted by confounders such as gender, age, tick bite (for tick-borne rickettsiosis) or contact with rodents (for typhus group rickettsiosis).

The Fisher's exact test was applied to evaluate the difference of clinical symptoms between groups positive and negative for rickettsiosis.

P-values < 0.05 were considered as statistically significant.

4. Results

4.1. Results of the tick study

In summary, in this study six different tick species originating from six sampling sites in two regions in Kazakhstan were investigated. The overview of collection sites and investigated tick species is presented on the Figure 4.1. This were 1193 *Ixodes persulcatus* (243 pools), 578 *Dermacentor marginatus* (129 pools), 470 *Haemaphysalis punctata* (104 pools), 77 *Hyalomma asiaticum* (17 pools), 14 *Dermacentor reticulatus* (3 pools) and 9 *Rhipicephalus turanicus* (5 pools). *I. persulcatus, D. marginatus* and *H. punctata* were the most abundant tick species in this PhD thesis.

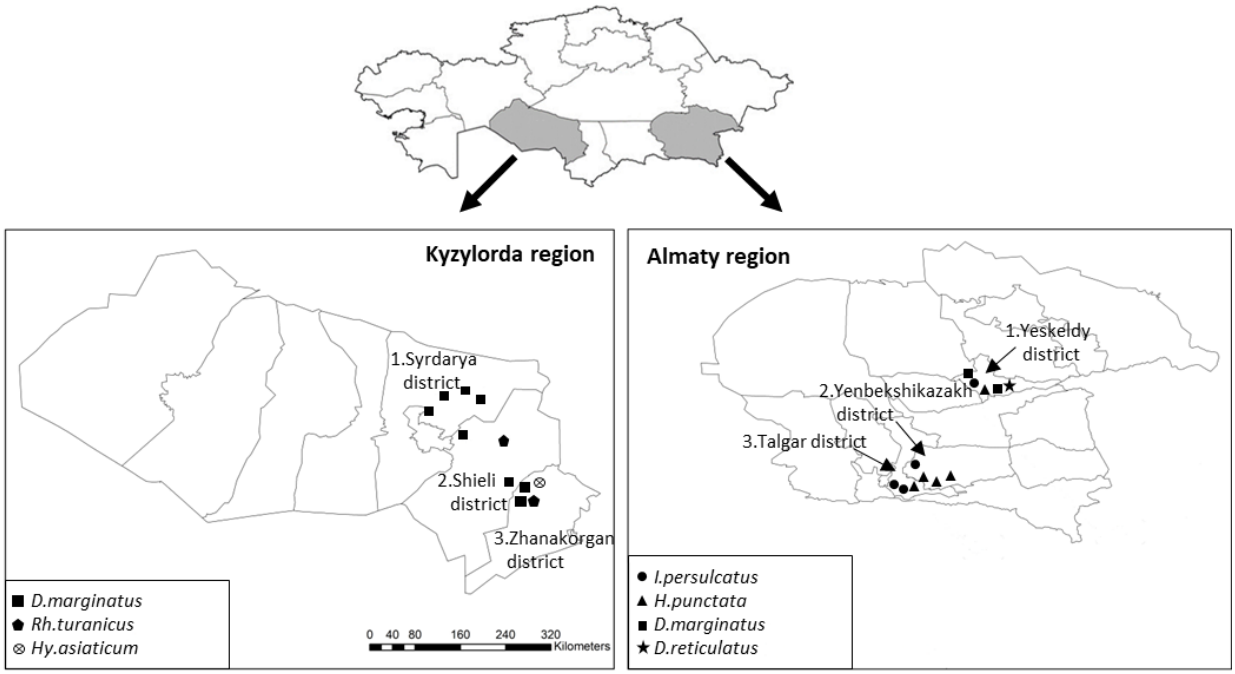

Figure 4.1 Kazakhstan, the two study regions and distribution of the tick species found at the sampling sites.

Four tick species out of six (*I. persulcatus, H. punctata, D. marginatus, D. reticulatus*) were found in Almaty region, with three of them (*I. persulcatus, H. punctata, D. reticulatus*) only in this region. Three tick species out of six (*Hy. asiaticum*, *Rh. turanicus, D. marginatus*) were detected in Kyzylorda region, with two of them (*Hy. asiaticum*, *Rh. turanicus)* only from this region. *D. marginatus* was sampled much more often in Kyzylorda region than in Almaty region (Table 4.1).

Table 4.1 Overview on collected tick species originating from two regions in Kazakhstan.

Localities (regions/districts)	Ticks	Pools	*I. persulcatus*		*H. punctata*		*D. marginatus*		*D. reticulatus*		*Hy. asiaticum*		*Rh. turanicus*	
			n	pools	n	pools	n	pools	n	pools	n	pools	n	pools
Almaty region:														
Talgar	505	104	504	103	1	1	0	0	0	0	0	0	0	0
Yeskeldy	709	148	610	123	25	7	60	15	14	3	0	0	0	0
Yenbekshikazakh	523	113	79	17	444	96	0	0	0	0	0	0	0	0
Sum Almaty	1737	365	1193	243	470	104	60	15	14	3	0	0	0	0
Kyzylorda region:														
Syrdarya	203	46	0	0	0	0	203	46	0	0	0	0	0	0
Shieli	202	46	0	0	0	0	199	43	0	0	0	0	3	3
Zhanakorgan	199	44	0	0	0	0	116	25	0	0	77	17	6	2
Sum Kyzylorda	604	136	0	0	0	0	518	114	0	0	77	17	9	5
Total	2341	501	1193	243	470	104	578	129	14	3	77	17	9	5

Abbreviations: *I., Ixodes; H., Haemaphysalis; D., Dermacentor; Hy., Hyalomma; Rh., Rhipicephalus*

The overall prevalence as per MIR of rickettsial DNA in the tick species and in the collecting localities (Almaty and Kyzylorda regions) was 42.3% (212/501; 95%CI; 37.9 – 46.8). The largest number of the Rickettsia partial *gltA* real-time PCR positive tick pools was determined in *Dermacentor* (128/132; 97.0%; 95%CI; 92.4 – 99.2) and *Haemaphysalis* (80/104; 76.9%; 95%CI; 66.6 – 83.8) genera collected from three selected districts of Kyzylorda region (between 56.8 to100%) and in Yenbekshikazakh district (79/113; 69.9%; 95%CI; 60.6 – 78.2) of Almaty region (Table 4.2). The smallest number of the *Rickettsia* partial *gltA* real-time PCR positive tick pools was found in *Ixodes* (3/243;1.2%; 95%CI; 0.3 – 3.6) and *Hyalomma* (1/17; 5.9%; 95%CI; 0.2 – 28.7) genera (Table 3) collected from Almaty region (1.9 – 11.5%) and in Zhanakorgan district (25/44; 56.8%; 95%CI; 41 – 71.7) of Kyzylorda region (Table 5). All *Rh. turanicus* from Zhanakorgan district of Kyzylorda region were negative in the screening real-time PCR.

Moreover, the MIR was calculated for each tick species and for both selected regions in this study (Table 4.2 and 4.3). A high MIR of Rickettsiae was detected in *Dermacentor* (MIR=21.4 – 21.6%) and *Haemaphysalis* (MIR=17.0%) ticks collected from Yenbekshikazakh district (Almaty region, MIR=15.1%) and from the three districts of Kyzylorda region (MIR=12.6 – 22.7%).

For details on tick numbers, collection sites and MIR see Table 4.2; 4.3 and Figure 4.2.

Table 4.2 Distribution of Rickettsial DNA in the collected tick species.

Tick species	*gltA* rtPCR positive [%] (number of *gltA* positive pools/total number of pools)	Number of ticks	MIR [%] (number of positive pools/ number of tested ticks)
Ixodes persulcatus	1.2 (3/243)	1193	0.3
Haemaphysalis punctata	76.9 (80/104)	470	17.0
Dermacentor marginatus	96.9 (125/129)	578	21.6
Dermacentor reticulatus	100 (3/3)	14	21.4
Hyalomma asiaticum	5.9 (1/17)	77	1.3
Rhipicephalus turanicus	0 (0/5)	9	0
Total	42.3 (212/501)	2341	9.1

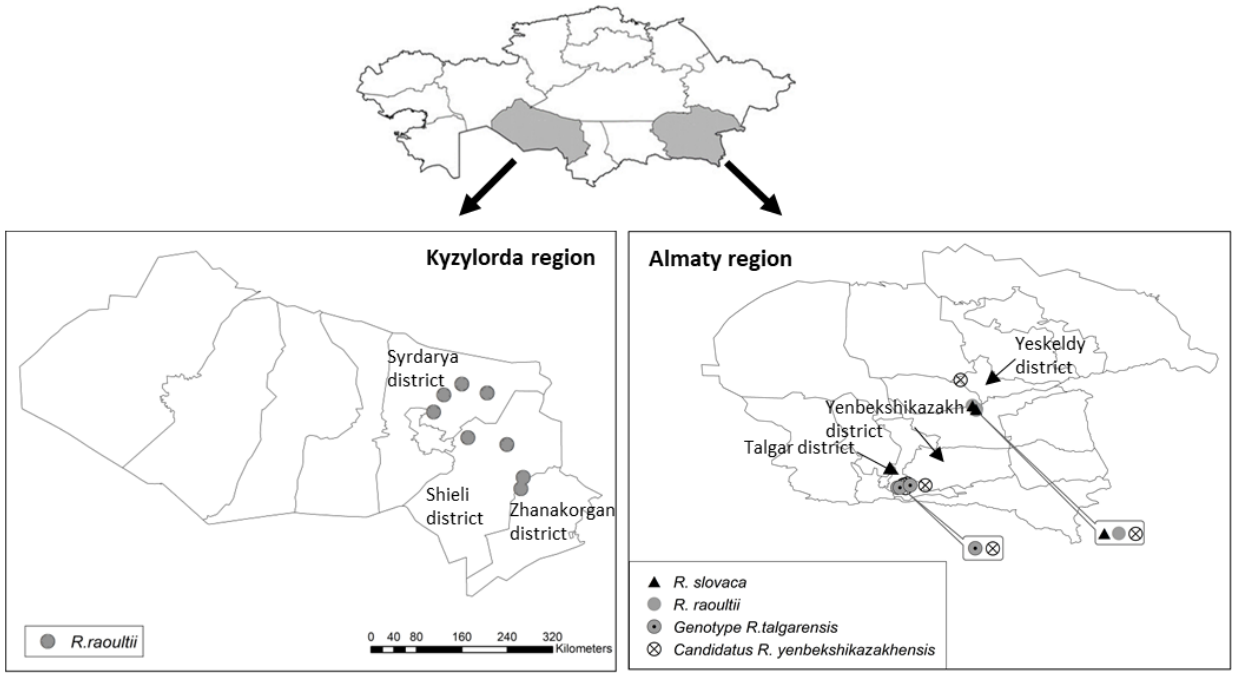

Figure 4.2 Kazakhstan, the two study regions and distribution of the detected *Rickettsia* species found at the sampling sites.

Table 4.3 Distribution of Rickettsial DNA in the collecting localities.

Localities (regions/districts)	*gltA* rtPCR positive [%] (number of *gltA* positive pools/total number of pools)	Number of ticks	MIR [%] (number of positive pools/ number of tested ticks)
Almaty region:			
Talgar	1.9 (2/104)	505	0.4
Yeskeldy (Tekeli city)	11.5 (17/148)	709	2.4
Yenbekshikazakh	69.9 (79/113)	523	15.1
Kyzylorda region:			
Syrdarya	100.0 (46/46)	203	22.7
Shieli	93.5 (43/46)	202	21.3
Zhanakorgan	56.8 (25/44)	199	12.6
Total	42.3 (212/501)	2341	9.1

As a result of MLST, Blast and afterwards phylogenetic analyses, for 209 of the 212 Rickettsia-positive samples the Rickettsia species could be determined. In summary four *Rickettsia* spp. were identified in the molecular investigations (Tables 4.4 – 4.6, Fig. 4.2). Two already known Rickettsia species – *R. raoultii* and *R. slovaca* – were identified by sequencing. *R. raoultii* was confirmed in 124 samples (124/209; 59.3%; 95%CI; 52.3 – 66.1) by sequencing partial *ompB (n=123),* partial *ompA* IV (n=9), *23S-5S* (n=9)*,* in *D. marginatus*, *D. reticulatus* and *Hy. asiaticum* from Kyzylorda region (n=113) and around Tekeli city in Almaty region (n=11). Further, *R. slovaca* (n=3 by partial *ompB* (n=2), partial *ompA*IV (n=1), *23S-5S* (n=2) genes) was detected in *D. marginatus* pools only around Tekeli city in Almaty region (3/209; 1.4%; 95%CI; 0.3 – 4.1), (Table 4.4 – 4.6, Figs. 4.3 – 4.8).

Table 4.4 Distribution of detected Rickettsia species in the tick species*.

Ticks	*R. raoultii*	*R. slovaca*	*Candidatus* R. yenbekshikazakhensis	*Genotype* R. talgarensis	Sum	Number of tick pools
I. persulcatus	0	0	0	3	**3**	**243**
H. punctata	0	0	80	0	**80**	**104**
D. marginatus	119	3	0	0	**122**	**129**
D. reticulatus	3	0	0	0	**3**	**3**
Hy. asiaticum	1	0	0	0	**1**	**17**
Rh. turanicus	0	0	0	0	**0**	**5**
Total	**123**	**3**	**80**	**3**	**209**	**501**

* for three samples no sequences were obtained, *R.*, *Rickettsia*; *I.*, *Ixodes*; *H.*, *Haemaphysalis*; *D.*, *Dermacentor*; *Hy.*, *Hyalomma*; *Rh.*, *Rhipicephalus*

Table 4.5 Distribution of Rickettsia species by collecting localities.

Localities (regions/districts)	*R. raoultii*	*R. slovaca*	*Canidatus* R. yenbekshikazakhensis	*Genotype* R. talgarensis	Sum	Number of tick pools
Almaty region:						
Talgar district	0	0	0	2	**2**	**104**
Yeskeldy district (Tekeli city)	11	3	2	0	**16**	**148**
Yenbekshikazakh district	0	0	78	1	**79**	**113**
Kyzylorda region:						
Syrdarya district	45	0	0	0	**45**	**46**
Shieli district	42	0	0	0	**42***	**46**
Zhanakorgan district	25	0	0	0	**25**	**44**
Total	**123**	**3**	**80**	**3**	**209**	**501**

* for three samples no sequences were obtained

Table 4.6 Sequences with 100% homology to known Rickettsia species.

Rickettsia spp.	*ompB*	*ompA*IV	*23S-5S*	*sca4*	*16S*	*gltA*	Total sequences
R. raoultii	123	9	9	nd	nd	nd	141
R. slovaca	2	1	2	nd	nd	nd	5
Total	125	10	11	nd	nd	nd	146

Abbreviation: nd, not determined as rickettsiae could be identified by sequences of other gene fragments

The new "*Candidatus* R. yenbekshikazakhensis" was confirmed in 80 samples (80/209; 38.2%; 95%CI; 31.7 – 45.2) and by MLST of the partial fragments of *ompB* (n=77), *ompA* IV (n=30), *23S-5S* (n=9)*, sca4* (n=34)*, 16S* (n=6)*, gltA* (n=35) genes (Table 4.4, 4.5, 4.7). "*Candidatus* Rickettsia yenbekshikazakhensis" has been identified in 16.0% of all 501 investigated tick pools (80/501; 95%CI; 12.9 – 19.5). "*Candidatus* R. yenbekshikazakhensis" was detected in high prevalence in *H. punctata* pools (80/104, 76.9%) from Yenbekshikazakh district (n=78) and around Tekeli city (n=1) in Almaty region, respectively. This "Candidatus" species has been detected only in one tick species (*H. punctata*) collected from two districts (Yeskeldy and Yenbekshikazakh districts) of Almaty region (Tables 4.4, 4.5, 4.7, Figs. 4.2 – 4.8).

We could further detect the new "genotype R. talgarensis" in three samples by analysis of the partial *ompA*IV (n=3), *23S-5S* (n=2)*, 16S* (n=2) genes in the ticks (3/209, 1.5%) in 0.6% of all 501 tested tick pools (3/501; 95%CI; 0.1 – 1.7) (n=2341). This genotype was only present in *I. persulcatus* received from two districts (Talgar and Yenbekshikazakh districts) in Almaty region (Tables 4.4, 4.5, 4.7, Figs. 4.2 – 4.8).

Unfortunately, one sample could not be sequenced and in two samples a mixture of different Rickettsia species was detected by sequencing of gene fragments. Overlapping chromatograms indicating a mixture of sequences were found for partial *ompA*IV, *gltA*, *16S* and *sca4* sequences for sample Kyzylorda 061 (*D. marginatus*, Kyzylorda region, Shieli district) and in the *ompB*, *ompA*IV and *gltA* sequences for sample Tekeli 076 (*D. marginatus*, Almaty region, Yeskeldy district), respectively.

Table 4.7 Overview of closest nucleotide identities of Candidatus species to the first hit in BLAST Rickettsia species.

Partial Genes	Maximum identity to known *Rickettsia* sp.	*Candidatus* R. yenbekshikazakhensis	*Genotype* R. talgarensis
ompB	>99.2	99.0% – CP013133, *R. rhipicephali*	n.a.
*ompA*IV	>98.8	99.0% – U83446, *R. aeschlimanni*	92.7% – CP003304, *R. canadensis*
23S-5S	n.a.	96.1% – AY125016, *R. aeschlimannii strain*	88.4% – CP003304., *R. canadensis*
16S	>99.8	99.8% – CP003319, *Ri. massiliae* 99.8% – HM050274, *R. aeschlimannii strain*	99.4% – CP003319.1 *R. massiliae*
sca4	>99.3	99.1% – HM050275, *R. aeschlimannii* * and 98.3% - HM050275, *R.aeschlimannii* °	n.a.
gltA	>99.9	100% – CP015012, *R. amblyommatis* 100% – KU723495, *R. aeschlimannii* 100% – KT588058, *R. massiliae*	n.a.

* all sequences from Yenbenshikazakh, ° sample Tekeli 093, n.a. no sequences available for comparison

All phylogenetic trees of the five different gene sequences are shown in Figure 4.3 – 4.8 in the following pages.

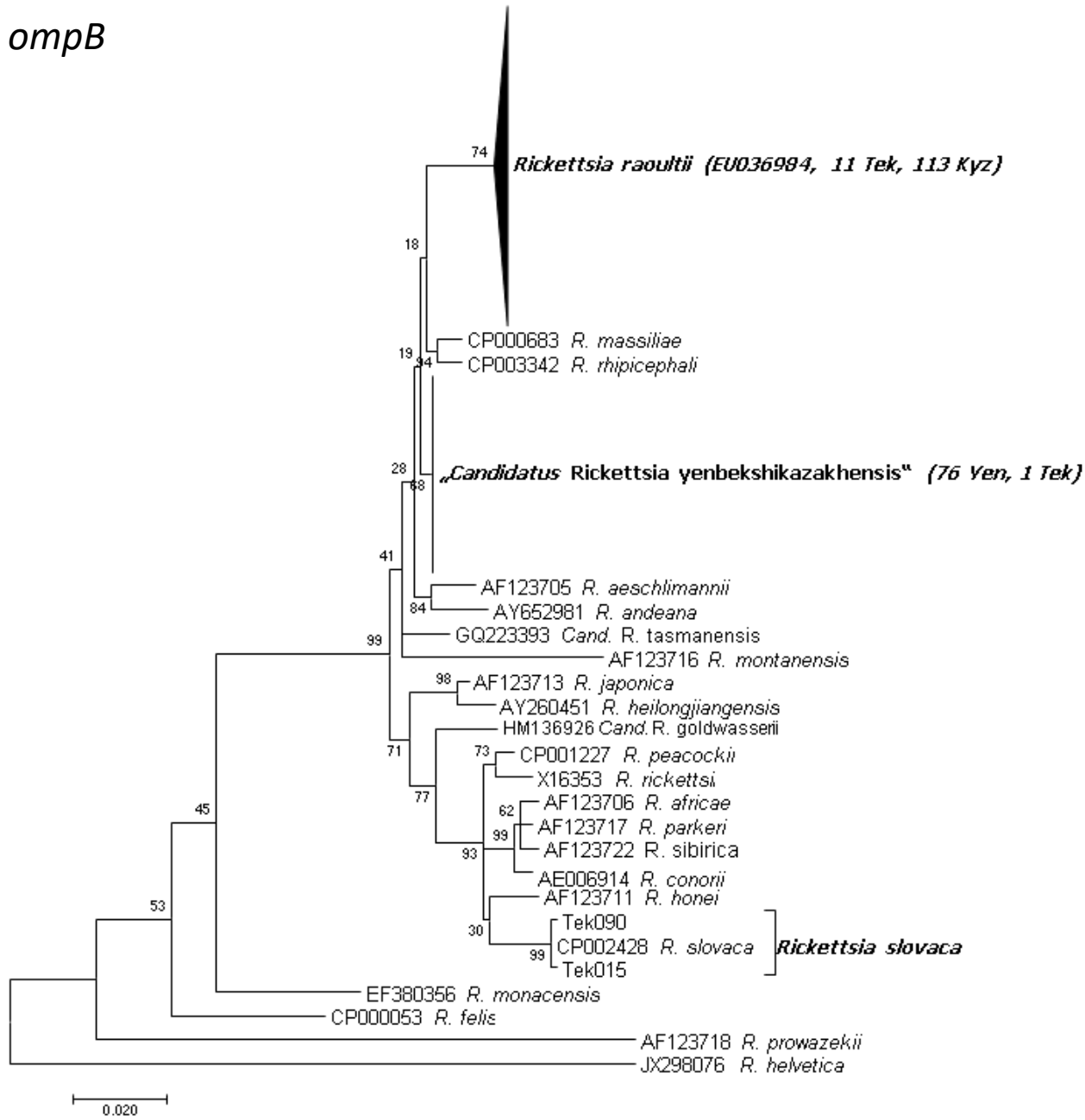

Figure 4.3 Maximum Likelihood phylogenetic tree, based on 226 partial *ompB* DNA sequences, with 203 sequences originating from amplificates from Kazakh tick DNA and 23 from the GenBank database. 124 sequences from Kazakh ticks were 100% identical to *R. raoultii*, two were 100% identical to *R. slovaca*, and 77 sequences formed a new cluster "*Candidatus* Rickettsia yenbekshikazakhensis" (76 sequences from Yenbekshikazakh district, 1 from Yeskeldy district - Tekeli city). The tree with the highest log-likelihood (-3541.6714) is shown. There were a total of 806 positions in the final dataset.

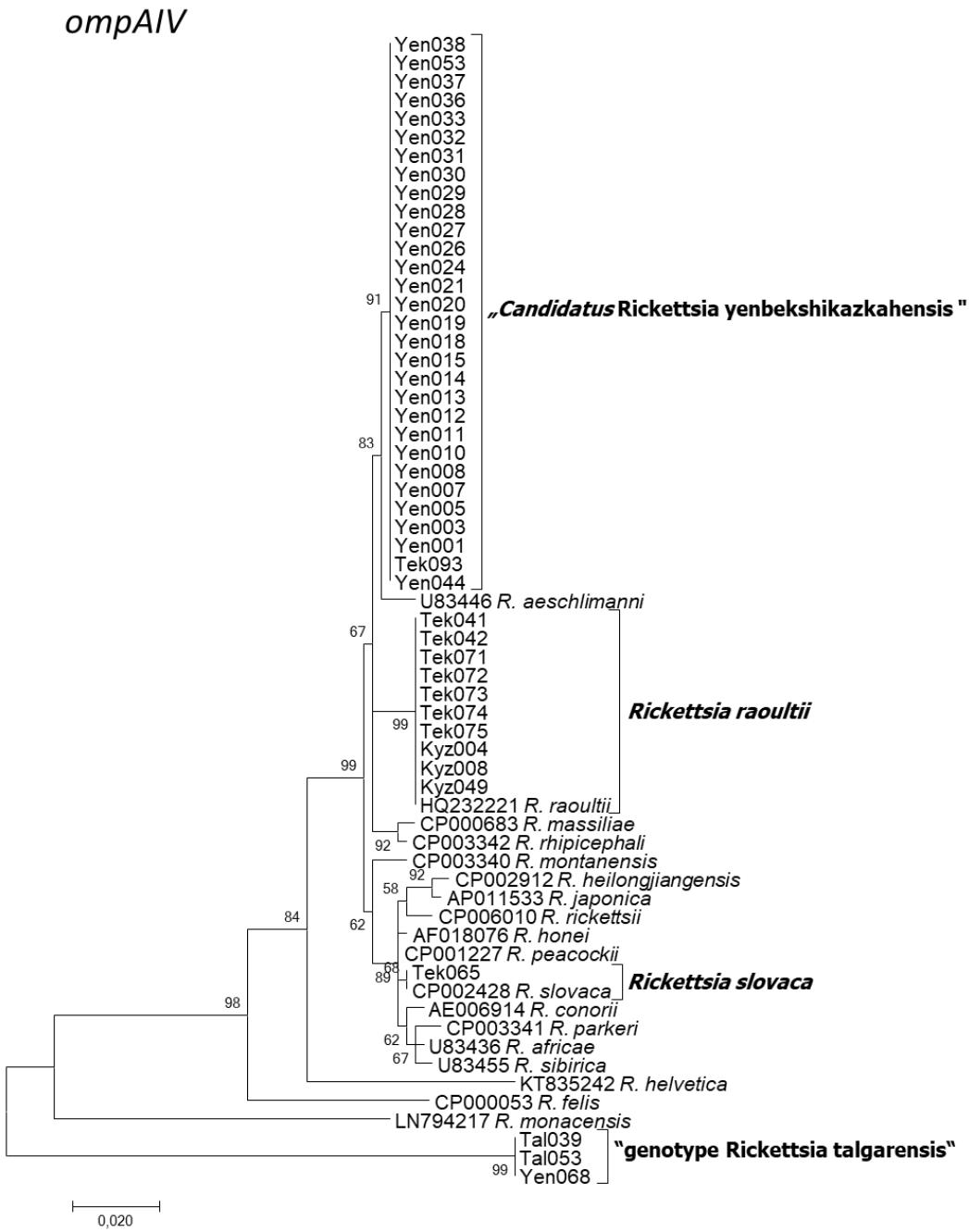

Figure 4.4 Maximum Likelihood phylogenetic tree, based on 62 partial *ompA*IV sequences, with 44 sequences originating from amplificates from Kazakh tick DNA and 20 from the GenBank database. 10 sequences from Kazakh ticks were 100% identical to *R. raoultii*, one sequence was identical to *R. slovaca*. 30 sequences formed a new cluster "*Candidatus* Rickettsia yenbekshikazakhensis" (29 sequences from Yenbekshikazakh district and 1 from Yeskeldy district around Tekeli city) and three a new cluster "genotype Rickettsia talgarensis" (1 sequence from Yenbekshikazakh

district, 2 from Yeskeldy district - Tekeli city). There were a total of 864 positions in the final dataset. The tree with the highest log-likelihood (-1803.5066) is shown.

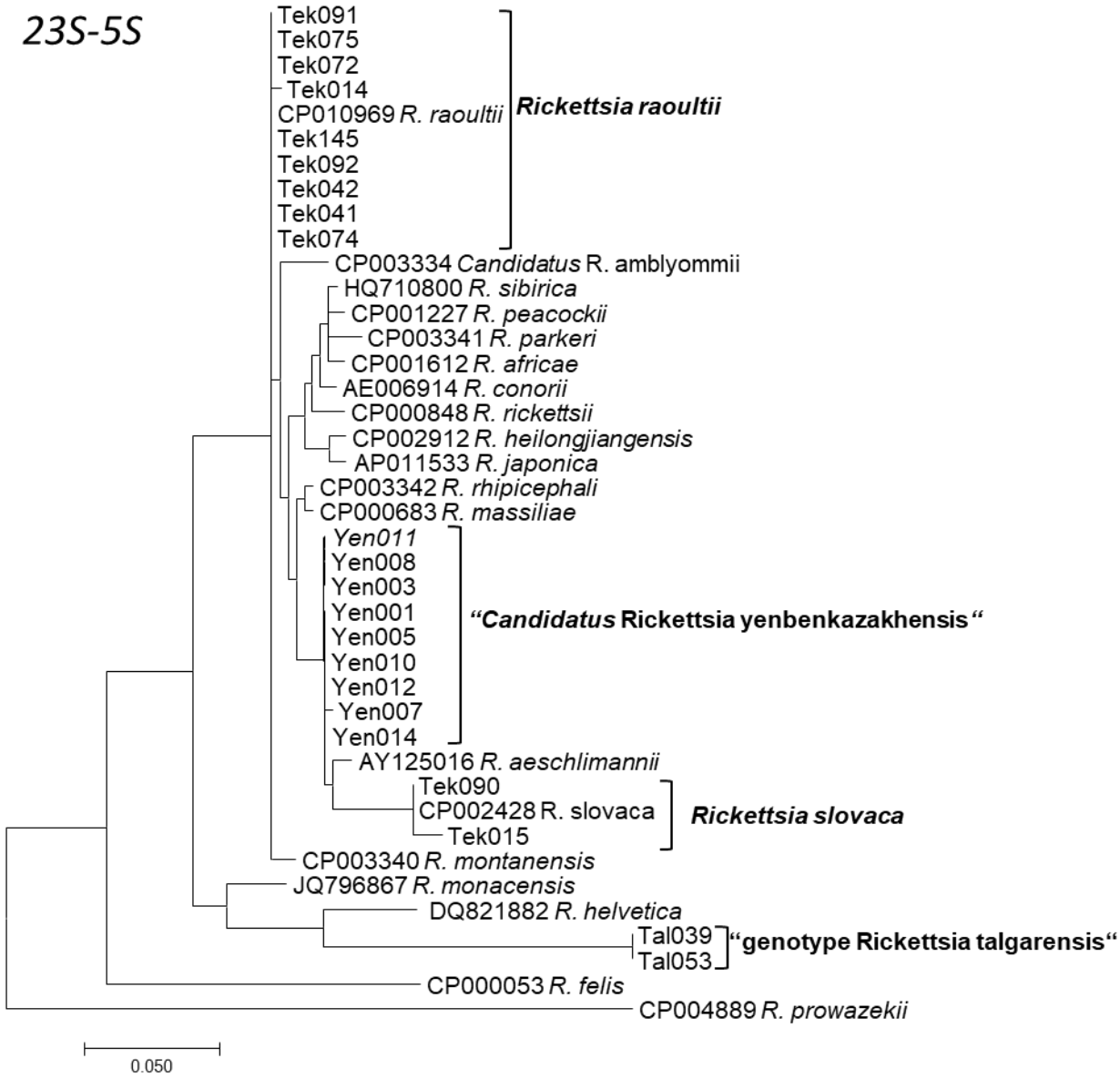

Figure 4.5 Maximum Likelihood phylogenetic tree, based on 40 partial 23S-5S sequences, with 22 sequences originating from Kazakh ticks and 18 from GenBank. Nine sequences from Kazakh ticks were 100% identical to *R. raoultii*, two sequences were identical to *R. slovaca*. Nine sequences Yenbekshikazakh district formed a new cluster "*Candidatus* Rickettsia yenbekshikazakhensis". There were a total of 367 positions in the final dataset. The tree with the highest log-likelihood (-1572.3294) is shown.

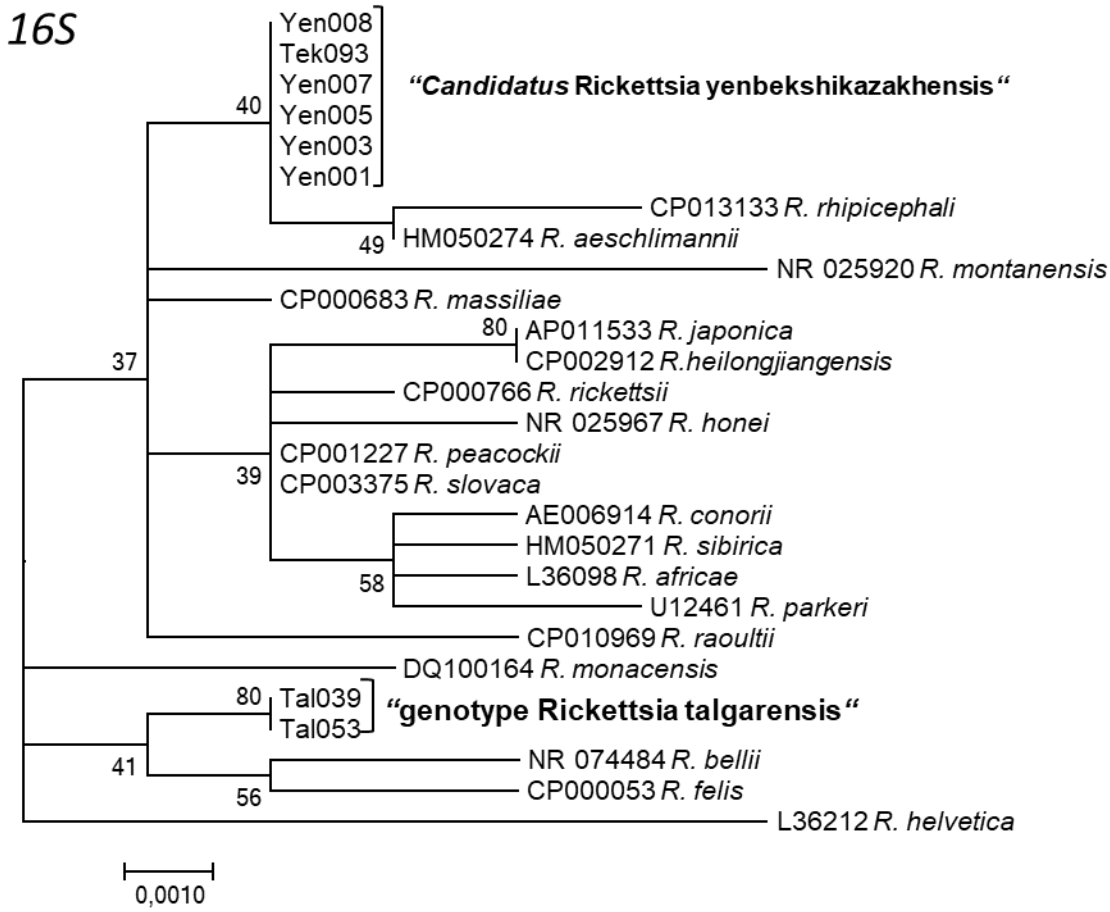

Figure 4.6 Maximum Likelihood phylogenetic tree, based on partial 27 partial *16S* sequences, with 8 sequences originating from Kazakh ticks and 19 from GenBank. Six sequences formed a new cluster "*Candidatus* Rickettsia yenbekshikazakhensis" (5 sequences from Yenbekshikazakh district, 1 from Yeskeldy district - Tekeli city) and two sequences from DNA of ticks from Tekeli the new cluster "genotype Rickettsia talgarensis". There were a total of 717 positions in the final dataset. The tree with the highest log-likelihood (-1287.3794) is shown.

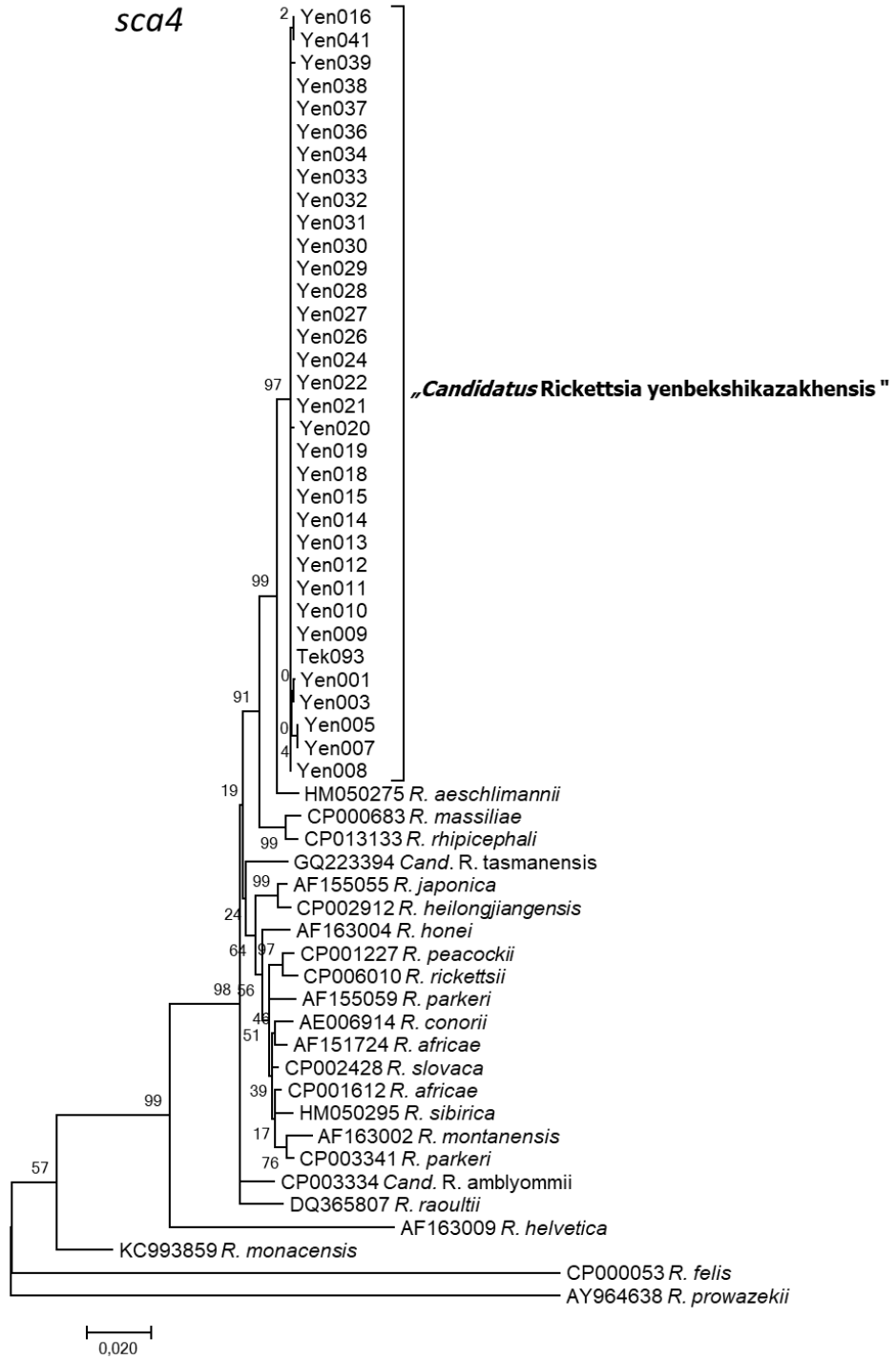

Figure 4.7 Maximum Likelihood phylogenetic tree, based on 57 partial *sca4* sequences with 34 sequences originating from Kazakh tick DNAs (33 from Yenbekshikazakh district, 1 from Yeskeldy district - Tekeli city) and 23 from GenBank. There were a total of 1.115 positions in the final dataset. The tree with the highest log-likelihood (-4809.7101) is shown.

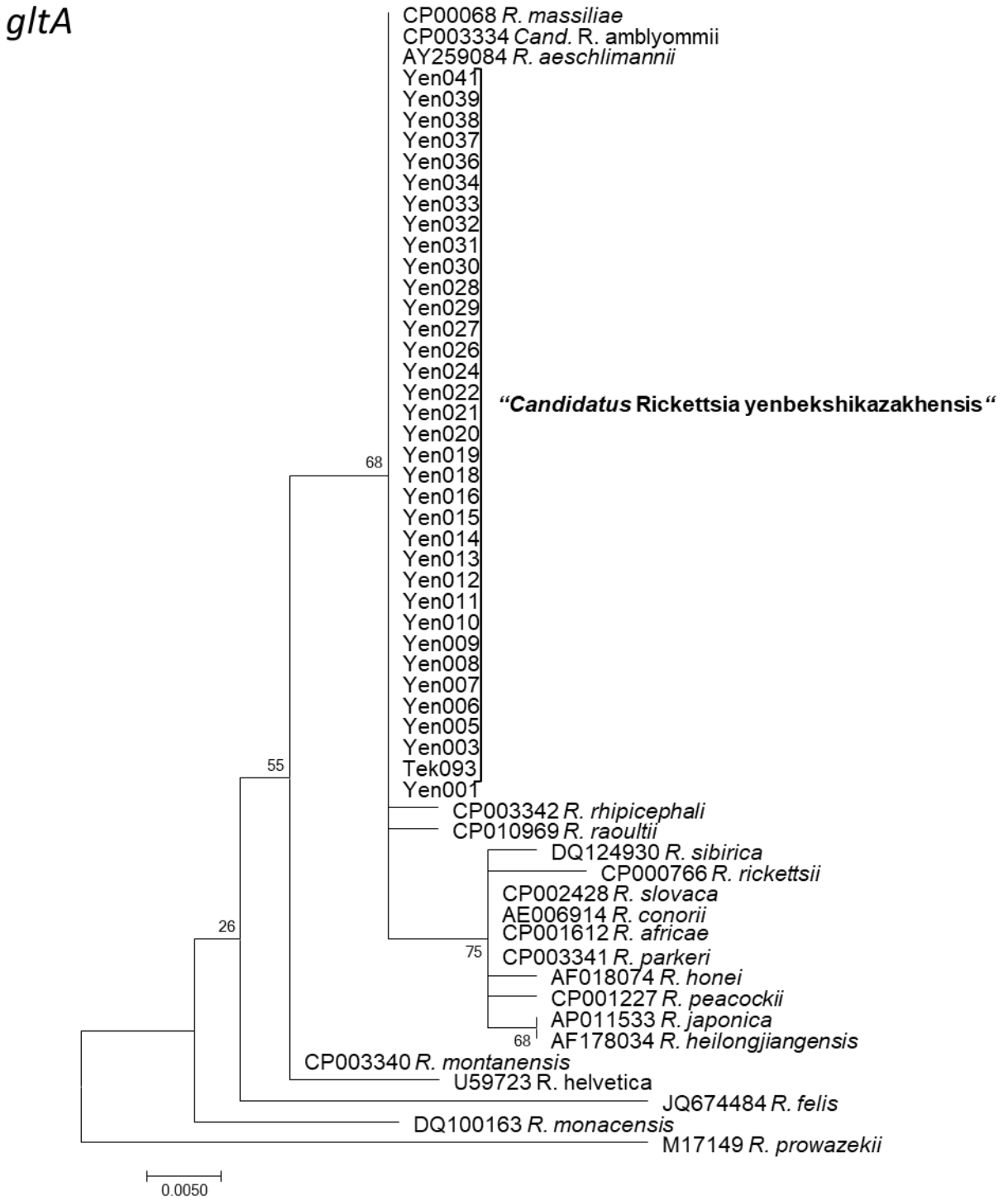

Figure 4.8 Maximum Likelihood phylogenetic tree, based on partial 55 partial *gltA* sequences, with 35 sequences originating from Kazakh tick DNAs forming the new "*Candidatus* Rickettsia yenbekshikazakhensis" (34 from Yenbekshikazakh district, 1 from Yeskeldy district around Tekeli city) and 20 from GenBank. There were a total of 318 positions in the final dataset. The tree with the highest log-likelihood (-641.7358) is shown.

4.2. Results of the serological study.

Totally 950 patients with FUO from 13 hospitals of the two selected regions were enrolled in this study between 2015 to 2016. Only 802 (84.4%) enrolees with FUO, out of these, had all the requirements to be included and investigated in this study (i.e. collected paired serum and completely filled questionnaires), the remaining 148 patients (15.6%) were excluded from the study.

Out of 802 paired serum samples, IgG antibodies to Rickettsia SFG were detected in 99 (26.2%; 95%CI; 22 – 30.9%) patients from Almaty region (n=378), in 151 (35.6%; 95%CI; 31.2 – 40.3%) patients from Kyzylorda region and totally in 250 (31.2%; 95%CI; 28.1 – 34.5%) patients from both regions (table 4.8).

Table 4.8 Prevalence of IgG antibodies against spotted fever group of Rickettsia in patients presenting with fever of unknown origin in two regions of Kazakhstan (2015-2016).

Localities (regions/hospitals)	Acute infection[1]	Previous infection[2]	Negative samples	Number of samples
Almaty region:				
Almaty	0	8 (6%)	125 (94%)	133
Taldykorgan	0	23 (52.3%)	21 (47.7%)	44
Tekeli	1 (1.2%)	30 (35.7%)	53 (63.1%)	84
Usharal	0	14 (58.3%)	10 (41.7%)	24
Yesyk	0	15 (25.0%)	45 (75.0%)	60
Kaskelen	0	8 (29.6%)	19 (70.4%)	27
Shelek	0	0	4	4
Kabanbay	0	0	1	1
Kapshagay	0	0	1	1
Sum	1 (0.3%)	98 (25.9%)	279 (73.8%)	378
Kyzylorda region:				
Kyzylorda	6 (2.3%)	90 (34.8%)	163 (62.9%)	259
Syrdarya	1 (2.2%)	9 (20.0%)	35 (77.8%)	45
Shieli	1 (2.0%)	27 (55.1%)	21 (42.9%)	49
Zhanakorgan	2 (2.8%)	15 (21.1%)	54 (76.1%)	71
Sum	10 (2.4%)	141 (33.2%)	273 (64.4%)	424
Total	11	239	552	802

[1] – shown by fourfold titer change in IgG

[2] – IgG positive sera samples including titrated samples

In summary 40 (5%; 95%CI; 4.7 – 6.7%) collected paired serum samples were titrated for IgG in order to distinguish previous and acute infections with SFG Rickettsia. In summary, 16 serum pairs had a low titer, eight serum pairs had a medium titer and 16 serum pairs had a high titer (for details see table 4.9). In eleven (1.4%; 95%CI; 0.8 –

2.4%) of the patients from the two selected regions a fourfold increase in IgG titer between first and second serum was detected by ELISA indicating an acute infection with SFG Rickettsia (table 4.10).

Table 4.9 Results of ELISA IgG titers against spotted fever group of Rickettsia.

IgG titer range (1st serum / 2nd serum)	Almaty region	Kyzylorda region	Total
Low titer (1:100 – 1:200/1:100 – 1:200)	6	10	16
Medium titer (1:100 – 1:400/1:400)	5	3	8
High titer (1:100 – 1:1600/1:800 – 1:3200)	4	12	16
Total	15	25	40

All eleven patients with the evidence of an acute spotted fever group rickettsiosis had a fever, headache and weakness (table 4.10). Nine of the eleven patients (81%) complained of exanthema. Six febrile inpatients (54.5%) reported swollen lymph nodes. Four enrolees (36.3%) had a muscle pain and only one had a neck pain.

Table 4.10 Patients with acute infection of the spotted fever group of Rickettsia.

Patient No	ELISA titers (1st/2nd serum)	Gender/Age	Exanthema	History of tick bite
Almaty region				
ESK-600 004	1:400 / 1:1600	f / 51	no	No
Kyzylorda region				
KYZ2-280 023	1:200 / 1:1600	m / 29	yes, muscle pain	No
KYZ2-280 051	1:100 / 1:800	m / 56	yes	Yes
KYZ2-280 052	1:100 / 1:400	f / 56	yes	Yes
KYZ2-280 156	1:100 / 1:800	f / 55	yes, enlarged lymph nodes	Yes
KYZ2-280 165	1:200 / 1:3200	f / 73	yes, enlarged lymph nodes	Yes
KYZ2-280 173	1:100 / 1:6400	m / 55	no, enlarged lymph nodes	Yes
SYR-250 004	1:200 / 1:1600	f / 66	yes, neck pain, muscle pain, enlarged lymph nodes	Yes
SHY-260 001	1:800 / 1:3200	m / 57	yes, muscle pain, enlarged lymph nodes	No
ZHA-270 001	1:100 / 1:3200	m / 57	yes, muscle pain, enlarged lymph nodes	No
ZHA-270 064	1:800 / 1:3200	f / 34	yes	No

Abbreviations: f – female, m – male

IgM antibodies against typhus group of Rickettsia were identified in 15 (1.9%; 95%CI; 1.1 – 3.1%) serum samples collected from Almaty and Kyzylorda regions (table 4.11). The fourfold titer increase in IgG antibodies between first and second sera was detected by ELISA in 7 (0.9%; 95%CI; 0.4 – 1.8%) patients with FUO from both regions that proves the presence of an acute infection with typhus group of Rickettsia (table 4.11). The previous infection with typhus rickettsiosis was distinguished by ELISA in 248 (30.9%; 95%CI; 27.8 – 34.2%) feverish cases from the two selected regions (table 4.11).

Table 4.11 Prevalence of IgM and IgG antibodies against typhus group of Rickettsia in patients presenting with fever of unknown origin in two regions of Kazakhstan (2015-2016).

Localities (regions/hospitals)	Previous infection[1]	Fourfold titer increase in IgG	IgM positive samples	Negative samples	Number of samples
Almaty region:					
Almaty	29 (21.8%)	0	2 (1.5%)	102 (76.7%)	133
Taldykorgan	29 (65.9%)	2 (4.6%)	3 (6.8%)	10 (22.7%)	44
Tekeli	42 (50.0%)	0	1 (1.2%)	41 (48.8%)	84
Usharal	16 (66.7%)	0	0	8 (33.3%)	24
Yesyk	28 (46.7%)	1 (1.7%)	0	31 (51.6%)	60
Kaskelen	14 (51.9%)	0	2 (7.4%)	11 (40.7%)	27
Shelek	2 (50.0%)	0	0	2 (50.0%)	4
Kabanbay	1 (100%)	0	0	0	1
Kapshagay	0	0	0	1 (100%)	1
Sum	161 (42.6%)	3 (0.8%)	8 (2.1%)	206 (54.5%)	378
Kyzylorda region:					
Kyzylorda	45 (17.4%)	1 (0.4%)	5 (1.9%)	208 (80.3%)	259
Syrdarya	7 (15.6%)	0	0	38 (84.4%)	45
Shieli	14 (28.6%)	1 (2%)	1 (2%)	33 (67.4%)	49
Zhanakorgan	21 (29.6%)	2 (2.8%)	1 (1.4%)	47 (66.2%)	71
Sum	87 (20.5%)	4 (0.9%)	7 (1.7%)	326 (76.9%)	424
Total	248	7	15	532	802

[1] – IgG positive sera samples including titrated samples with a titer difference lower than four-fold

Also, due to identify acute rickettsial typhus infection, 49 (6.1%; 95%CI; 4.7 – 8%) collected serum pairs from both regions were titrated for the presence of IgG antibodies and 33 paired sera had a low titer, 14 paired sera had a medium titer and two paired sera had a high titer (table 4.12).

Table 4.12 Results of ELISA IgG titers against typhus group of Rickettsia.

IgG titer range (1st serum / 2nd serum)	Almaty region	Kyzylorda region	Total
Low titer (1:100 – 1:200/1:100 – 1:200)	20	13	33
Medium titer (1:100 – 1:400/1:400)	9	5	14
High titer (1:100 /1:800)	1	1	2
Total	30	19	49

Almost all of the patients with acute typhus rickettsiosis had unspecific clinical symptoms such as fever (100%), weakness (90.9%), headache (86.4%), muscle pain (31.8%), enlarged lymph nodes (31.8%) and neck pain (27.3%). The exanthema, which is the main specific rickettsial typhus symptom, was observed in 40.9% of acute cases. For summary see table 4.13.

Table 4.13 Patients with acute infection of the typhus group of Rickettsia[1].

Patient No	IgM reactive	ELISA titers (1st/2nd serum)	Gender/Age	Symptoms	Contact with rodents
Almaty region					
ALM-800 012	+	–	m / 25	fever, headache, weakness, exanthema	No
ALM-800 048	+	–	m / 21	fever, headache, neck pain, weakness, enlarged lymph nodes	No
TALD-900 002	+	–	m / 34	fever, headache, neck pain, weakness, exanthema	No
TALD-900 029	+	–	m / 38	fever, headache	No
TALD-900 033	+	–	m / 32	fever, headache, neck pain, weakness	No
TALD-900 036	-	1:100 – 1:400	m / 35	fever, neck pain, weakness, muscle pain, enlarged lymph nodes	Yes[2]
TALD-900 040	-	1:100 – 1:400	m / 32	fever, headache, weakness	No
ESK-600 017	+	–	m / 43	fever, headache, weakness	Yes[2]
YEN1-200 069	-	1:100 – 1:800	m / 19	fever, neck pain	No
KAS-700 001	+	–	m / 67	fever, weakness	No
KAS-700 016	+	–	f / 42	fever, headache, weakness, muscle pain	No

Kyzylorda region					
KYZ2-280 003	-	1:100 – 1:800	f / 61	fever, headache, weakness, exanthema	Yes[2]
Kyz2-280 023	+	–	m / 29	fever, headache, weakness, muscle pain, exanthema	No
Kyz2-280 041	+	–	m / 56	fever, headache, weakness, muscle pain, exanthema	No
Kyz2-280 116	+	–	m / 19	fever, headache, weakness, enlarged lymph nodes	No
SYR-250 095	+	–	m / 27	fever, headache, neck pain, weakness, enlarged lymph nodes	No
SYR-250 106	+	–	m / 34	fever, headache, weakness, enlarged lymph nodes	No
SHY-260 001	-	1:100 – 1:400	m / 57	fever, headache, weakness, muscle pain, enlarged lymph nodes, exanthema	No
SHY-260 010	+	–	m / 24	fever, headache, weakness	No
ZHA-270 001	-	1:100 – 1:400	m / 57	fever, headache, weakness, muscle pain, enlarged lymph nodes, exanthema	Yes[2]
ZHA-270 038	+	–	m / 69	fever, headache, weakness, muscle pain, exanthema	No
ZHA-270 060	-	1:100 – 1:400	f / 60	fever, headache, weakness, exanthema,	No

[1] – shown by IgM and fourfold titer change in IgG, «+» – ELISA IgM positive patients

[2] – rarely come into contact with rodents, f – female, m – male

Among enrolees with acute spotted fever group rickettsiosis from Almaty and Kyzylorda regions (n=11), predominate patients aged 46 to 75 years (table 4.14). The median age of these inpatients from both regions was 56 years. Table 4.14 gives an overview on demographic, epidemiological and clinical characteristics of the patients with acute tick-borne rickettsiosis in two regions of Kazakhstan.

There was defined a significant difference in age groups in tick-borne rickettsiosis positive results scores. Logistic regression analysis revealed an age older 55 years as an independent predictor for this disease (odds ratio for 56-65 years age

group 6.01, CI 95%: 1.54:20.42; p = 0.005; odds ratio for 66-75 years age group 7.03, CI 95%: 1.04:29.16; p = 0.02).

Table 4.14 Revealed features of inpatients with acute infection* of the spotted fever group of rickettsia in two regions of Kazakhstan (2015 – 2016)

Factor		Number of samples (n=799)	Positive samples (n=11)	Logistic regression model			
				Univariate analysis		Multivariate analysis	
				OR (95% CI)	p-value	OR a**(95% CI)	p-value
Gender	Female	302	6	Reference	-	-	-
	Male	497	5	0.5 (0.14-1.68)	0.26	0.58 (0.17-1.98)	0.39
Age, years	15-25	291	0	NAp	NAp	NAp	NAp
	26-35	199	2	0.67 (0.1-2.61)	0.61	0.73 (0.15-3.45)	0.69
	36-45	121	0	NAp	NAp	NAp	NAp
	46-55	87	3	3.14 (0.68-11.1)	0.1	2.51 (0.64-9.83)	0.19
	56-65	**72**	**4**	**6.05 (1.55-20.56)**	**0.005**	**5.24 (1.47-18.73)**	**0.01**
	66-75	**26**	**2**	**7.07 (1.04-29.36)**	**0.02**	4.39 (0.84-22.94)	0.08
	>75	3	0	NAp	NAp	NAp	NAp
Tick bite	No	620	5	Reference	-	-	-
	Yes	**179**	**6**	**4.27 (1.27-14.96)**	**0.02**	2.9 (0.84-10.03)	0.09
Place of residence							
Urban area		422	6	Reference	-	-	-
Rural area		377	5	0.93 (0.27-3.12)	0.91	0.75 (0.22-2.57)	0.65
Contacts with wild animals							
No		788	10	Reference	-	-	-
Yes		11	1	7.78 (0.4 – 46.92)	0.06	4.71 (0.46-48.28)	0.19
Nature trip							
No		594	9	Reference	-	-	-
Yes		205	2	0.64 (0.1-2.51)	0.57	0.6 (0.12-2.98)	0.53
Garden work							
No		273	2	Reference	-	-	-
Yes		526	9	2.36 (0.6-15.54)	0.27	1.4 (0.29-6.91)	0.68
Livestock availability							
No		440	4	Reference	-	-	-
Yes		359	7	2.17 (0.65-8.33)	0.22	1.71 (0.48-6.13)	0.41
Current occupation							
Students		113	0	NAp	NAp	NAp	NAp
Plants farmer		13	1	6.47 (0.34-38.06)	0.09	4.8 (0.5-45.95)	0.17
Animals farmer		15	0	NAp	NAp	NAp	NAp
Forestry farmer		8	1	11.16 (0.57-71.7)	0.03	7.79 (0.63-96.62)	0.11
Keeping the house		32	0	NAp	NAp	NAp	NAp
Unskilled labourer		**54**	**3**	**5.42 (1.16-19.38)**	**0.01**	**8.1 (1.84-35.69)**	**0.006**
Skilled labourer		132	1	0.49 (0.03-2.59)	0.49	0.74 (0.09-6.16)	0.78
Driver		27	1	3.33 (0.18-18.49)	0.26	4.93 (0.52-46.35)	0.16
Adm/acad. professional		63	1	1.17 (0.06-6.26)	0.88	1.74 (0.2-15.05)	0.61
Businessman/woman		25	0	NAp	NAp	NAp	NAp
Nurse/physician/pharmacist		20	1	4.05 (0.22-22.79)	0.19	4.33 (0.47-39.6)	0.19
Unemployed							

Retiree	163	1	0.39 (0.02-2.04)	0.37	0.39 (0.05-3.12)	0.37
Military person	38	1	2.03 (0.11-11.02)	0.51	0.17 (0.02-1.64)	0.12
Declined_to_answer	50	0	NAp	NAp	NAp	NAp
NA	26	0	NAp	NAp	NAp	NAp
	23	0	NAp	NAp	NAp	NAp

* shown by four fold titer change in IgG

**OR – odds ratio; OR a – odds ratio adjusted for gender, age and tick bite rate; CI – confidence interval; NA – not available; NAp – not applicable

The chance to observe a positive tick-borne rickettsiosis in the group bitten by a tick is 4.33 times higher than in the group of not bitten persons (CI95%: 1.28:15.18; p = 0.017). However, the association between the disease and a tick bite is strongly confounded by age due to the prevalence of a rickettsiosis positive result is much higher in older age groups. Therefore, after adjustment for age the association between a tick bite and disease is no longer significant (OR = 2.91, CI 95%: 0.84:10.08; p = 0,092).

Our analysis revealed statistically significant association between work in forestry and chance of positive results for rickettsiosis (OR = 11.1, CI 95%: 0.57:71.24; p = 0.03), moreover, the risk group was unskilled labourers (OR = 5.38, CI 95%: 1.15:19.25; p = 0.02).

Almost half of the febrile participants showing an acute SFG rickettsiosis (n=5) are males. The same pattern we have found with a place of residence: five patients are living in a rural area and six patients in the urban area. The majority of inpatients with acute tick-borne rickettsiosis (81.8%, n=9) didn’t have any trip to the nature during the last month. At the same time most of them (72.7%, n=8) did a daily garden work. Six enrolees out of eleven (%) had a tick bite during the past month. In 63.6% of acute cases (n=7), daily contact with livestock was observed (table 4.14).

Patients with acute infection (n=11) had not only unspecific mild clinical symptoms such as fever (100%), headache (100%), weakness (81.8%) but also some distinctive signs as enlarged regional lymph nodes (54.5%) and exanthema (81.8%) (table 4.15).

Table 4.15 Clinical manifestations in patients with acute infection* of the spotted fever group of rickettsia.**

Symptoms	OR (CI 95%)	p-value
Neck pain	0.29 (0.01 – 2.09)	0.31
Weakness	4.34 (0.61 – 189.02)	0.19
Muscle pain	2.31 (0.49 – 9.23)	0.24
Lymphadenopathy	1.82 (0.46 – 7.61)	0.36
Exanthema	**16.28 (3.32 – 156.08)**	**0.00004**

* shown by four fold titer change in IgG

** data based on Fisher's exact test; OR – odds ratio; CI – confidence interval

The demographic, epidemiological and clinical characteristics of the acute cases with rickettsial typhus in two regions of Kazakhstan are presented in table 4.16 and 4.17. Among the participants with acute infection of the typhus group Rickettsia (n=22), the persons aged between 15 to 35 years (n=12). The median age of these inpatients from both regions was 34.5 years.

The majority of feverish patients with acute rickettsial typhus infection (81.8%, n=18) are males. Less than half of these enrolees (45.5%, n=10) are living in a rural area. In many acute cases the contacts with wild animals (95.6%, n=21), with rodents (81.8%, n=18) and trip to the nature (68.2%, n=15) didn't observe. The 59.1% of acute patients (n=13) have a daily contact with their livestock (table 4.16).

Table 4.16 Revealed features of inpatients with acute infection* of the typhus group of Rickettsia in two regions of Kazakhstan (2015 – 2016).

Factor		Number of samples (n=799)	Positive samples (n=22)	Logistic regression model			
				Univariate analysis		Multivariate analysis	
				OR (95% CI)	p-value	OR a**(95% CI)	p-value
Gender	Female	302	4	Reference	-	-	-
	Male	497	18	2.8 (1.03-9.76)	0.06	3.05 (1.02-9.16)	0.05
Age, years	15-25	291	5	0.5 (0.16-1.29)	0.18	0.46 (0.16-1.27)	0.13
	26-35	199	7	1.42 (0.54-3.42)	0.45	1.49 (0.59-3.71)	0.4
	36-45	121	3	0.88 (0.2-2.64)	0.84	0.85 (0.25-2.92)	0.79
	46-55	87	0	NAp	NAp	NAp	NAp
	56-65	**72**	**5**	**3.12 (1-8.17)**	**0.03**	**3.37 (1.19-9.58)**	**0.02**
	66-75	26	2	3.14 (0.48-11.62)	0.14	3.58 (0.78-16.46)	0.1
	>75	3	0	NAp	NAp	NAp	NAp
Contact with rodents							
	No	696	18	Reference	-	-	-
	Yes	103	4	1.07 (0.25-3.21)	0.92	0.87 (0.25-3.04)	0.83
Place of residence							
	Urban area	422	12	Reference	-	-	-
	Rural area	377	10	0.93 (0.39-2.18)	0.87	0.89 (0.37-2.13)	0.79
Contacts with wild animals							
	No	788	21	Reference	-	-	-
	Yes	11	1	3.65 (0.19-20.42)	0.23	2.98 (0.34-26.39)	0.33
Nature trip							
	No	594	15	Reference	-	-	-
	Yes	205	7	1.36 (0.51-3.29)	0.5	1.26 (0.5-3.17)	0.62
Garden work							
	No	273	9	Reference	-	-	-
	Yes	526	13	0.74 (0.32-1.82)	0.5	0.64 (0.27-1.54)	0.32
Livestock availability							
	No	440	9	Reference	-	-	-
	Yes	359	13	1.49 (0.63-3.56)	0.36	1.43 (0.6-3.39)	0.41
Current occupation							
	Students	113	0	NAp	NAp	NAp	NAp
	Plants farmer	13	1	3.04 (0.16-16.53)	0.3	1.96 (0.23-16.43)	0.54
	Animals farmer	15	0	NAp	NAp	NAp	NAp
	Forestry farmer	8	0	NAp	NAp	NAp	NAp
	Keeping the house	32	0	NAp	NAp	NAp	NAp
	Unskilled labourer	54	1	0.65 (0.04:3.18)	0.68	0.6 (0.08-4.54)	0.62
	Skilled labourer	132	3	0.79 (0.18-2.37)	0.71	0.76 (0.22-2.65)	0.67
	Driver	27	2	3.01 (0.46-11.1)	0.15	2.06 (0.44-9.59)	0.36
	Adm/acad. professional	63	3	1.89 (0.43-5.74)	0.32	2.39 (0.67-8.52)	0.18
	Businessman/woman	25	0	NAp	NAp	NAp	NAp
	Nurse/physician/pharmacist	20	0	NAp	NAp	NAp	NAp
	Unemployed	163	6	1.48 (0.52-3.67)	0.42	1.57 (0.6-4.13)	0.36
	Retiree	38	3	3.35 (0.76-10.42)	0.06	2.42 (0.46-12.64)	0.3
	Military person	50	2	1.52 (0.24-5.41)	0.58	1.87 (0.38-9.29)	0.44

Declined_to_answer	26	1	1.43 (0.08-7.29)	0.73	0.89 (0.11-7.09)	0.91
NA	23	0	NAp	NAp	NAp	NAp

*shown by IgM and four fold titer change in IgG

**OR – odds ratio; OR a – odds ratio adjusted for gender, age and contact with rodents; CI – confidence interval; NA – not available; NAp – not applicable

Table 4.17 Clinical manifestations in patients with acute infection* of the typhus group of Rickettsia.**

Symptoms	OR (CI 95%)	p-value
Neck pain	1.12 (0.35 – 3.06)	0.81
Weakness	**4.4 (1.05 – 39.11)**	**0.03**
Muscle pain	1.9 (0.64 – 5.06)	0.18
Lymphadenopathy	0.7 (0.24 – 1.84)	0.51
Exanthema	2.47 (0.91 – 6.36)	0.06

* shown by IgM and four fold titer change in IgG

** data based on Fisher's exact test; OR – odds ratio; CI – confidence interval

5. Discussion

5.1. Discussion of tick study

The spotted-fever group of rickettsiae in tick study was for the first time a large-scale and comprehensive investigation performed in two selected pilot regions of Kazakhstan. The two regions have different landscapes and this is the reason that in both selected regions several different tick species have been flagged. *Dermacentor marginatus* is the most abundant tick typically found at the collection sites in the desert and semi-desert landscape of Kyzylorda region (16) which is the classical habitat of this tick species (37, 38). However, the three selected collection sites in Almaty region is a mountainous landscape covered with forest (Almaty region of Kazakhstan. http://zhetysu.gov.kz/ru/o-regione/) which are the typical habitats for Ixodes ticks (37) having in Almaty region the highest abundance of all flagged tick species (48.1%). In Almaty region the highest variability of tick species with five tick species (*I. persulcatus, H. punctata, D. marginatus, D. reticulatus*) was found.

The taxonomic classification of ticks investigated in this study was done using morphologic markers (17-21). *D. marginatus* and *D. niveus* were summed up to *Dermacentor marginatus* as the Editor of Parasites and Vectors stated that these are conspecific. Genetic markers give a hint on that statement, however detailed data are lacking (39-41).

The results of my studies clearly show that five of the seven collected tick species carry rickettsial DNA. In Kyzylorda region where Dermacentor species are the main species, 56.8-100% of the ticks' pools were Rickettsia positive, and only *R. raoultii* was found in the two species of Dermacentor, *D. marginatus* and *D. reticulatus*. Interestingly at the three tick flagging sites in Almaty region, which has been considered so far as a non-endemic region, four Rickettsia species are present, indicating the highest variability of Rickettsia in ticks. In 59% of the tick pools *R. raoultii* and in three pools *R. slovaca* were found – the important point is that these two species are pathogenic for humans. The obtained data indicate that the main vectors of these two pathogens are Dermacentor ticks, which is in line with data from neighboring countries i.e. Russian Federation, Mongolia or northwestern China which is located close to Almaty region of Kazakhstan (7, 42 – 50). An important point of my study is, that *R. raoultii* was for the first time detected in one *Hy. asiaticum* tick pool collected from Kyzylorda region of Kazakhstan.

R. raoultii and *R. slovaca* are well described human pathogens that cause the "SENLAT" (scalp eschar and neck lymphadenopathy) syndrome manifested by scalp eschars and neck lymphadenopathy, TIBOLA (tick-borne lymphadenopathy) or DEBONEL (*Dermacentor*-borne necrosis erythema lymphadenopathy) after a tick bite (7, 51). The high Minimum Infection Rate of *R. raoultii* in the studied ticks and a recent case study in China were 26 cases of *R. raoultii* infections with varying severity were published (52), indicate that *R. raoultii* should be included in the diagnosis of rickettsioses in Kazakhstan. The occurrence of *R. slovaca* was before described in the sheep ked *(Melophagus ovinus)* sampled in the localities of Xinjiang Uygur Autonomous Region (northwestern China), that is neighboring the Almaty region of Kazakhstan (48). My studies show that *R. slovaca* is present in Kazakhstan – so further studies are needed to clarify the role of this tick for diseases in humans and to check if it is abundant in other regions in Kazakhstan.

I describe for the first time a new Candidatus Rickettsia in Kazakhstan, the "*Candidatus* R. yenbekshikazakhensis" was confirmed by MLST of six gene fragments. For the *ompB, 23S-5S, 16S* and *sca4* but not for the *ompAIV* and *gltA* it fulfills the criteria of Fournier et al., (2003) (8) to designate it as a new "*Candidatus*" species (table 4.7). It has been suggested to taxonomically classify Rickettsia as new "*Candidatus*" if at least four or five sequences are newly described (4, 8–10). The closest species to "*Candidatus* R. yenbekshikazakhensis" is *R. massiliae* which is also known to be human pathogenic and to induce SENLAT syndrome (53, 54). The new "*Candidatus* R. yenbekshikazakhensis" was detected in two regions and in 87.6% of all investigated *H. punctata* ticks, which indicates that this tick species might be its main vector.

Further "genotype R. talgarensis" was detected in three tick pools. The analysis of three gene fragments, *ompAIV, 23S-5S* and *16S* could be performed showing a quite high divergence to all known Rickettsiae (table 4.7). The detected agent fulfills therefore the criteria to be describe as new genotype (8). For both, "*Candidatus* R. yenbekshikazakhensis" and "genotype R. talgarensis" further studies are needed to clarify their role as pathogen for humans.

5.1. Discussion of serological study

The serological study described in this thesis was designed as a cross sectional investigation of inpatients with fever of unknown origin hospitalized in two selected southern regions of Kazakhstan. It is the first study to examine the causative agents of fever of undetermined origin in Kazakhstan. This study was focused on both rickettsial diseases – tick-borne rickettsiosis and typhus group rickettsiosis – as a cause of acute febrile response. We demonstrated that the typhus group rickettsia in comparison to tick-transmitted rickettsia two times more can be the cause of the fever of obscure origin in hospital-enrolled patients from Almaty and Kyzylorda regions of Kazakhstan.

This study had a number of limitations. 802 out of 950 feverish inpatients donated 1st and 2nd serum samples, complete questionnaires and the prevalence of acute tick-bite rickettsiosis among febrile patients was based only on the detection of the four-fold IgG titer differences in the 2nd serum samples. The prevalence of acute spotted fever group rickettsiosis in Almaty region was much lower than that observed among febrile enrolees from Kyzylorda region. This obtained data once again prove the endemicity of Kyzylorda region for this rickettsial disease in comparison with Almaty region. The prevalence of acute typhus group rickettsiosis in our study was based on the detection of IgM in the 1st sera and the four-fold titer changes in IgG of the 2nd serum samples. The prevalence of this acute rickettsial disease in Almaty region was similar to that observed among inpatients with undetermined hyperthermia in Kyzylorda regions.

This is the first comprehensive study on both groups of Rickettsia as a potential to cause the fever of unknown origin in humans. The received data shows that tick-borne rickettsiosis and rickettsial typhus are present in Kazakhstan. Most of the Kazakh general clinicians are not familiar with the symptoms caused by rickettsial diseases and therefore many cases probably remain undiagnosed in our country. Therefore, it is very important to raise awareness of these emerging diseases in Kazakhstan.

6. Conclusion

6.1 Conclusion

In this thesis I made first in depth investigations on tick borne Rickettsia in ticks and as cause of FUO and for the first-time generated data on Rickettsia of the typhoid group -associated FUO.

In the first part of this thesis I describe the first, very detailed investigation of Rickettsia in ticks and for the first time MLST of the positive found samples. I found out, that the prevalence expressed as MIR of SFG rickettsial DNA in the tick species and in the collecting localities (Almaty and Kyzylorda regions) was with 42.3% quite high. Further in Dermacentor (97.0%) and Haemaphysalis (76.0%) genera collected from three selected districts of Kyzylorda region (between 56.8 to100%) and in Yenbekshikazakh district (79/113; 69.9%; 95%CI; 60.6 – 78.2) of Almaty region there were tremendous prevalences of SFG rickettsial DNA. In addition, I describe for the first-time details on Rickettsia species in ticks. Interestingly four Rickettsia species were found and most exciting two of these are unknown Rickettsia species, which was confirmed by several rickettsia DNA fragments. Remarkably Almaty region was so far not seen as endemic region for SFG Rickettsia.

Additionally, in the second part this thesis contains comprehensive data on SFG and murine typhus-Rickettsia specific antibodies in patients suffering on FUO. Despite we only could get 802 paired sera samples and questionnaires for SFG we IgG antibodies to Rickettsia SFG in 26.2% of the patients from Almaty region and in 35.6% from Kyzylorda region, with an average percentage lying at 31.2%. Acute SFG infections were detected in both oblasts in patients with FUO, with symptoms classically fund in different SFG rickettsiosis. In addition, I describe for the first time the presence of murine typhus in Kazakhstan, which is officially not registered in my country. These findings are of importance and I could even show that not only previous but also acute infections are present in over 6.1% of investigated samples from patients with FUO.

In summary the results of this thesis show that spotted fever and murine typhus might be of higher concern as thought before and that FUO has to be taken into account.

6.2 Recommendations

The clinical cases of tick-borne rickettsioses, which were registered by using CFT over the past 20 years in Kazakhstan, are so far not confirmed by other serological methods such as ELISA and by pathogen detection (e.g. rickettsial DNA by PCR). With the rising evidence on the relevance of rickettsiae in human infections and for improving epidemiological data, routine laboratory diagnostic tools must be implemented in all reporting laboratories in Kazakhstan. Our data also indicate that clinicians should be aware of SENLAT syndrome which is caused by two confirmed pathogens (*R. raoultii* and *R. slovaca*) circulating in the territory of Almaty and Kyzylorda regions. The present data indicate that tick-borne rickettsiae and associated pathological conditions in humans should be further investigated in all regions of Kazakhstan to estimate the importance and clinical impact caused by all four described rickettsiae.

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8. Annex

List of publications in International peer-reviewed journals

1. **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, 2019 (article in press)
2. 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.
3. 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.

Statement on Pre-release and Contribution

Two scientific articles from this study were prepared for publication. The first article has already been accepted for publication to the target journal “Parasites and Vectors” and is in the process of production. The title of the accepted article is “Prevalence of *Rickettsia* species in ticks including identification of unknown species in two regions in Kazakhstan”.

The second article entitled “Seroprevalence of rickettsiae in hospital-enrolled patients with fever of unknown origin in the southern regions of Kazakhstan” is presently under correction by co-authors for the submission to Parasites and Vectors journal.

The PhD candidate collected the tick and serum samples for investigation and with the help of trained medical doctors and sanitary specialists of medical facilities from pilot regions and Scientific Practical Centre for Sanitary Epidemiological Expertise and Monitoring. All laboratory work including tick sorting, homogenization, sample inactivation, DNA extraction, conducting real-time PCR, conventional PCR, ELISA tests and received data analysis were done by the PhD candidate under the close support of the direct supervisor. PhD candidate also wrote two articles and PhD thesis under the close supervision of all supervisors.

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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).

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 Date (dd/mm/yyyy)
Informed Consent Discussion

_____

Printed Name of Witness (if applicable)

_____ _____

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

Informed Consent Form

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 Date (dd/mm/yyyy)
Informed Consent Discussion

_____

Printed Name of Witness (if applicable)

_____ _____

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

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	Always lived in this place
		☐2	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 ☐	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

		☐ 6 ☐ 7 ☐ 8 ☐ 9 ☐ 10 ☐ 11 ☐ 12 ☐ 13 ☐ 14	☐ 10 ☐ 11 ☐ 12 ☐ 13 ☐ 14	Administrative or academic professional [3] Businessman/woman Nurse/Physician/Clinician/Pharmacist 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 products without pasteurization?	☐1 ☐2	No Yes If yes, from which animal? _____ _	

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 ☐6	Unusual movement Respiratory symptoms Gastroenterological symptoms Lesions Others, please specify: _____ Don't know			
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		Always/daily	Most of the times	Rarely	Never

	following animals? *("Contact" means working with these animals, milking, slaughtering, handling raw meat), but **NOT** eating cooked meat*					
	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	☐1 ☐2	Rural area Urban area

	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	Fever Headache Neck pain (meningism)

		☐4	Weakness of muscels 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 threat
		☐17	Congestion of nose
		☐18	Enlarged lymph nodes
		☐19	Icterus
6.3	Body temperature (*ear measurement*)	☐1	< 37.5°C
		☐2	> 37.5°C
			If body temperature is > 37.5°C, than fill out 6.4
6.4	Duration of high body temperature	☐1	< 3 days
		☐2	3 days and more
6.5	Blood pressure	☐1	Normal
		☐2	Hypotension
		☐3	Hypertension
6.6	Pulse rate	☐1	< 80
		☐2	80 – 100
		☐3	> 100
6.7	Skin conditions	☐1	Exanthema
		☐2	Ulceration
		☐3	Edema
		☐4	Others (*please specify*) _____
6.8	Stool	☐1	Diarrhoea
		☐2	Blood in stool
		☐3	Bright stool
		☐4	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