SCIENTIFIC REPORT

RealLine Rickettsia

Detection of Genetic Markers for Pathogens of Tick-Borne Rickettsioses by PCR Using "RealLine *Rickettsia species"* and "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis"* Assay Kits

More than 1 Million of people in Europe and Asia, including more than 500,000 people in Russia sought medical attention after a tick bite, being afraid of tick-borne infections (TBI) in 2017 [1]. The natural TBI foci demonstrate stability and a tendency to expand their habitats, which contributes to an increase in the number of infected people [2]. The most prevalent TBI is ixodes tick-borne borreliosis (ITB), however occurrence of other tick-borne infections, including rickettsiosis (aTBR), increases dramatically. Due to the lack of vaccines against rickettsioses, these diseases currently pose a serious problem for many countries [3–7].

The TBR pathogens are rickettsia, which occupy an intermediate position between viruses and bacteria and all belong to the tick-borne spotted fever (TBSF) group, which includes 19 different species. Seven of them have been found within the Russia, Finland, Austria, Germany: *R. conorii*, *R. sibirica*, *R. heilongjiangensis*, *R. slovaca*, *R. aeschlimannii*, *R. helvetica*, *R. raoultii* [7–10].

Currently, two rickettsioses of the TBSF group are officially registered in Russia: Astrakhan spotted fever (ASF) caused by *R. conorii subsp.caspiensis* and Siberian tick typhus (STT) caused by *R. sibirica* infection. Patients with ASF can develop severe forms of the disease with possible lethal outcome, while STT is characterised by a less severe course of infection [5, 11]. In 2017, the fraction of ASF in the total tick-borne rickettsioses morbidity in the RF was less than 10%, while STT accounted for about 80% [1].

Pathogenic rickettsia can be found in TBR endemic regions of Mediterranean, the Far East, Africa, and America [7].

A common reference to STT in scientific literature is North Asian tick-borne rickettsiosis (NATBR), which better corresponds to the modern concept of this disease [13]. Publications devoted to TBI also use the term Far-Eastern tick-borne rickettsiosis (FETBR), that was first described in Khabarovsk region and Primorsky kray (both – Russia), and later in other regions of Russia - Irkutsk region and in Altay [3, 5, 14–16]. While the pathogen for FETBR is *R. heilongjiangensis*, the clinical signs of the disease have no significant differences from NATBR, and it is also subject to official registration as STT.

The only presented diagnostic kit on the market is "Rickettsia Conoril EIA IgG/IgM" (Vircell S.L., Spain), but it has all disadvantages common for ELISA kits (sensitivity, ability to test antibodies after 7-10 days etc.) [7].

For patients bitten by a tick and suffering from fever, if no inoculation escharor eruption are present, it is difficult to differentiate TBR from TBE, ITB, and tick-borne relapsing fever caused by *Borrelia miyamotoi* infection. Thus, upon a complete examination of 37 patients with the preliminary STT diagnosis it was found that, in two cases, the fever was caused by *B. miyamotoi*, whose DNA was detected in the patients' blood [20].

The last decade has seen an explosive growth in the studies on the use of polymerase chain reaction (PCR) for TBI diagnostics. It has been demonstrated that, due to its high sensitivity, the method can detect extremely low levels of genetic material of pathogens for such diseases as TBE, ITB, babesiosis, anaplasmosis, ehrlichiosis in ticks, as well as in certain clinical samples from the patients. PCR had been successfully used for rickettsia DNA analysis in a number of studies in various laboratories [5, 14, 15, 21–25]. The detection of rickettsia DNA in blood or other clinical samples from the examined patient provides grounds for confirming the tick-borne rickettsiosis.

The study aims at the extended testing of the "RealLine DNA *Rickettsia species"* assay kit and "RealLine DNA *Rickettsia sibirica/Rickettsia heilongjiangensis"* multiplex kit for real-time PCR with fluorescence detection.

Materials and Methods. "RealLine *Rickettsia species*" kit uses primers and probes providing amplification and detection of a conservative region of gene *gltA* in all rickettsia species of the TBSF group. The second kit, "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis",* uses a fragment of gene *ompB* for *R. sibirica* and a fragment of gene *ompA* for *R. heilongjiangensis*, which can be produced in the same tube and independently detected in the real time in channels HEX and ROX, respectively, as the amplification targets.

For performing assays with these assay kits, 5123 ticks were collected, which belong to ten species circulating within nine regions of the Russia. 3243 ticks were collected by flagging in Novosibirsk, Irkutsk, Amur regions, Krasnoyarsky kray, Khabarovsky kray, Primorsky kray, the Republic of Altay, and Sakhalin island (Russia), 1670 specimens were picked off the bitten residents of Novosibirsk region, and 210 ticks were collected in Crimea when combing domestic animals. Tick homogenization to obtain their suspensions was performed as described in [30].

For testing the assay kits, we also used total DNA extracted from a set of clinical samples (biopsies and swabs collected from the inoculation eschar, whole blood, leucocyte blood fraction (LBF), plasma, and urine) from 264 patients bitten by a tick, who were admitted to healthcare facilities in the Republic of Altay and the city of Khabarovsk with suspected TBI.

Samples of whole blood, plasma and leucocyte fraction were prepared using the techniques published in Yakovchits et al. [31].

Collectionof swabs from the inoculation eschar. A skin crust at the inoculation eschar surface was soaked with saline for 1–2 minutes using a cotton swab and the remaining skin was removed. The swab was prepared using 350 µl of saline in a 1.5 ml tube (Eppendorf type). A cotton tampon soaked with solution from the tube was passed 5–7 times over the eschar surface gently penetrating inside the lesion with the tip. The liquid from the tampon was squeezed against tube walls and the procedure was repeated. The tampon was placed at the bottom of the tube, the upper part of the bud was cut or broken off to close the tube with its lid. Tube contents were shaken for 20–30 seconds, and the tube was centrifuged to collect any drops.

Total DNA for real-time PCR was extracted with the "RealLine Extraction 100" assay kit (BIORON Diagnostics, Germany) using 100 µl of the following samples: suspension of each tick, biopsy eschar homogenate and swab, blood plasma, LBF, or 50 µl of the whole blood. If the blood volume was increased to 250 µl, the contained erythrocytes were lysed using 1.5 ml of solution included with the "RealLine Hemolytic" assay. DNA was extracted from the resulting pellet as described in the instruction manual for the "RealLine Extraction 100" assay kit.

***Extracting Total DNA from Urine*.** A tube containing 10–12 ml of morning portion of urine was centrifuged for 10 minutes at 3000 rpm. The upper layer was carefully removed to leave a denser urine fraction with the volume of 1 ml at the bottom, from which total DNA was extracted using the "RealLine Extraction 1000" assay kit (BIORON Diagnostics, Germany). The instruction manual for the assay kit was amended to cover its use with urine samples. After addition of the concentrating solution to the sample, stirring and exposing for 10–15 minutes, the mixture was centrifuged for 5 minutes at 13,000 rpm (instead of 3000 rpm as per the instruction manual).The DNA extraction from the formed precipitate was conducted in accordance with the instruction manual for the assay kit, using 100 µl of the eluting solution (instead of the required 200 µl) at the last step to extract DNA from the sorbent.

The real-time PCR was performed on thermocyclers with real-time fluorescence detection of the block-type CFX96 (Bio-Rad, USA) and rotor-type Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (Qiagen, Germany). The nucleic acids samples for PCR had a volume of 50 µl.

The complete study of the clinical samples was performed using the following assay kits: "RealLine TBEV", "RealLine *Borrelia burgdorferi s.l.*", "RealLine *Anaplasma phagocytophilum/Ehrlichia muris*, *Ehrlichia chaffeensis"*, "RealLine DNA *Borrelia miyamotoi*" (BIORON Diagnostics, Germany).

To identify the species of rickettsia detected in the tested ticks and in certain clinical samples from patients, nucleotide sequences were determined for the following gene fragments: *gltA*, *ompA*, *ompB*, *Sca4*. The assays were performed on an automatic sequencer ABI Prism 3100 Genetic Analyser (Applied Biosystems, USA). The sequencing results were compared against the nucleotide sequences for rickettsia DNA contained in GenBank.

Results and Discussion. To determine the species diversity of rickettsia whose DNA is detected using the "RealLine *Rickettsia species"* assay kit, the assay kit was employed to study total DNA extracted from suspensions of eight tick species (Table 1). The positive real-time PCR samples were sequenced for 3–4 genes (*gltA*, *ompA*, *ompB*, *Sca4*). It was found that DNA of rickettsia in the tested ticks belong to 11 different species; 7 of them are pathogenic (*R. conorii, R. sibirica, R. heilongjiangensis, R. slovaca, R. aeschlimannii, R. massilae, R. mongolotimonae*), 2 are opportunistic pathogens (*R. raoultii* and *R. helvetica*), while *Candidatus R. tarasevichiae*, *Candidatus R. rara* are candidates for new rickettsia species with unproved pathogenicity. The circulation of two species, *R. massilae* and *R. mongolotimonae*, in the Crimean peninsula was reported for the first time.

Table 1: Results of detection of DNA of various rickettsia species with the "RealLine DNA *Rickettsia species*"assay kit and subsequent sequencing of positive PCR samples

Tick Species	Collection Site (all – in Russia)	Number of Ticks (%)		
		Sampling	Containing Rickettsia DNA	Containing Rickettsia species determined by DNA sequencing
D. nuttalli	Krasnoyarsky kray Minusinsk district	80	15 (18.8)	*R. raoultii*—10 (12.5) *R. sibirica*—5 (6.3)
H. concinna	Amur region, Magdachinsk district	72	15 (20.8)	*R. heilongjiangensis*—14 (19.4) *Candidatus R. rara*—1 (1.4)
I. persulcatus	Novosibirsk, suburbs	50	20 (40.0)	*Candidatus R. tarasevichiae*—20 (40.0)
I. persulcatus	Yuzhno-Sakhalinsk, suburbs	119	82 (68.9)	*R. helvetica*—76 (63.9) *Candidatus R. tarasevichiae*—6 (5.0)
R. sanguineus	Sevastopol, suburbs	50	14 (28.0)	*R. conorii*—8 (16.0)* *R. massilae*—2 (4.0)*
D. marginatus	The Republic of Crimea, Belogorsk district	40	25 (62.3)	*R. slovaca*—5 (12.5)* *R. raoultii*—18 (45.0)*
H. punctata	The Republic of Crimea, Saks district	100	11 (11.0)	*R. aeschlimannii*—7 (7.0)*
H. marginatum	The Republic of Crimea, Saks district	20	4 (20.0)	*R. mongolotimonae*—4 (20.0)

Thus, it was shown that use of the "RealLine *Rickettsia species"* assay kit allows the genetic material of all tick-borne rickettsiosis pathogens that can be found within Russia to be detected.

The "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis"* multiplex assay kit was developed for simultaneous detection of DNA of two rickettsia species officially attributed to STT pathogens, that account for about 80% of the total TBR morbidity in our country. This assay kit is intended for the detection of *R. sibirica* and *R. heilongjiangensis* in epidemiologic surveys in the endemic regions for testing ticks whose feeding on a

patient led to consequences, to predict STT development, and for assaying clinical samples from a patient to confirm this diagnosis.

Table 2 shows the results of testing 2614 ticks of six species: *D. nuttalli*, *D. silvarum*, *D. reticulatus*, *H. concinna*, *I. persulcatus*, *H. japonica* from five regions of Siberia and the Far East using the "RealLine DNA *Rickettsia species"* and "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis*" assay kits. During testing of the total DNA extracted from tick suspensions using the first of these assay kits, genetic material of rickettsia was found in 1439 (55.1 %) samples. In their subsequent additional testing using the "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis"* multiplex assay kit, DNA of these STT pathogens was detected in 177 cases (6.8 % of all tested ticks). The rate of tick infection with rickettsia in the examined samplings varied from 7.9 to 98.3 %; however, the corresponding indicator for pathogenic species was significantly lower (0.6–29.3 %). On the other hand, in the majority of the tested ticks the DNA copy number for these TBR pathogens varied in the range of 3.8×10^5–3×10^6, which suggests a high level of their infection with pathogenic rickettsia.

Table 2: Data from examination of 2614 ticks using the "RealLine *Rickettsia species"* and "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis*" assay kits

Collection Site	Number of Ticks (%)		
	Examined Species	Containing Rickettsia DNA	Containing DNA of Pathogenic Species
The Republic of Altay	*D. nuttalli—219* *D. silvarum—112* *D. reticulatus—128* *H. concinna—41* *I. persulcatus—82*	96 (43.8) 93 (83.0) 52 (41.4) 12 (29.3) 58 (71.0)	*R. sibirica—4 (1.8)* *R. heilongjiangensis—1 (0.9) Not found* *R. heilongjiangensis—12 (29.3)* *R. heilongjiangensis—2 (2.4)*
Krasnoyarsky region	*D. nuttalli—310*	97 (31.3)	*R. sibirica—53 (17.1)*
Irkutsk region	*D. nuttalli—499* *H. concinna—89* *I. persulcatus—76*	440 (88.2) 7 (7.9) 66 (86.7)	*R. sibirica—13 (2.6)* *R. heilongjiangensis—7 (7.9)* *R. heilongjiangensis—2 (2.6)*
Khabarovsky region	*H. concinna—72* *H. japonica—50*	18 (25.0) 9 (18.0)	*R. heilongjiangensis—18 (25.0)* *R. heilongjiangensis—2 (4.0)*
Amur region	*H. concinna—492* *I. persulcatus—272* *D. silvarum—172*	70 (14.2) 252 (92.6) 169 (98.3)	*R. heilongjiangensis—57 (11.6)* *R. heilongjiangensis—2 (0.7)* *R. sibirica—1 (0.6)* *R. heilongjiangensis—3 (1.7)*

The obtained data suggest that two species of STT pathogens circulate in the Republic of Altay, Irkutsk, and Amur regions: *R. sibirica* are found in ticks *D. nuttalli, D. silvarum*, while *R. heilongjiangensis* are found in arthropods *H. concinna, I. persulcatus*, and *D. silvarum.* The maximum *R. heilongjiangensis* morbidity of 29.3% was found in ticks of species *H. concinna* collected in the Republic of Altay; the *R. sibirica* infection rate in *D. nuttalli* ticks of Krasnoyarsk region was 17.1 %. In six districts of this region, the rate of *R. sibirica* DNA detection varied from 0 to 30 %, which indicates the presence of foci for these pathogenic rickettsia and its uneven territorial distribution among the circulating ticks. A similar focal distribution of STT pathogens was also observed in other examined regions.

S.N. Shpynov et al. reported the detection of *R. sibirica* DNA in ticks *I. persulcatus* [32]. However, the results of our study of all ticks of this species from eight regions of Siberia and the Far East (including those collected in regions where genetic material of rickettsia was detected in *D. nuttalli* ticks) were negative. The ticks *I. persulcatus* are known for their elevated aggression towards humans [33, 34]. For this reason, although

the infection rate of these ticks with *R. heilongjiangensis* in the Republic of Altay, Irkutsk and Amur regions is only 0.7–2.6 %, they can play a role in the dissemination of pathogenic rickettsia in the endemic regions and in TBR infection of humans upon feeding of an infected parasite.

In earlier studies, DNA of pathogenic rickettsia was found in ticks *D. silvarum*, *D. reticulatus*, and *I. persulcatus* caught in several populated sites of Novosibirsk region [32], where occasional STT cases had been registered. Therefore, we tested 1670 samples of total DNA extracted from ticks, which bit residents of the city of Novosibirsk and its suburbs over the period of 2014 to 2017. Genetic material of *R. sibirica* was found in two cases, but the species of the tick could not be determined. Such a low occurrence rate for this rickettsia species in this region is probably the reason for the low STT infection rate in Novosibirsk region, in contrast to the Republic of Altay and Krasnoyarsky kray.

Using the "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis"* assay kit, 221 ticks *H. concinna* and 87 specimens of *H. japonica* collected in Primorskykraywere tested. As a result of testing the samples of total DNA extracted from the pooled ticks (2–3 specimens), genetic material of *R. heilongjiangensis* was detected in 9 out of 82 (11.1%) pools of *H. concinna* and in 3 out of 29 (10.3%) pools of *H. japonica*, while *R. sibirica* DNA was not found in any of the tested samples. It appears that the ticks of these species do not carry the mentioned pathogenic rickettsia in Primorsky region, as opposed to *D. silvarum*, *D. nuttalli*, *D. marginatus* [16].

New assay kits were also used to test the clinical samples from patients with the preliminary STT diagnosis and for patients with the suspected TBI development. The efficiency of PCR detection of pathogenic rickettsia DNA depends significantly on the type of the tested clinical sample used for testing. Rickettsiosis is characterised by a low number of pathogens in blood circulation of a patient and a rather limited period of their circulation in the fever period. Therefore, attempts to detect rickettsia DNA in blood serum of an infected person often yield negative results, especially in cases where the blood was collected not at the peak of fever, but in the period of lowered temperature [24]. A biopsy from the site of inoculated eschar or erupted skin is considered the most suitable material for PCR, but its collection requires a surgical intervention. Recently, swabs collected from the inoculation eschar were suggested as alternative to biopsy [7]. We used this clinical material from 83 persons suffering from a tick bite in the city of Gorno-Altaisk for extraction of total DNA and its parallel analysis with the "RealLine *Rickettsia species*" and "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis"* assay kits to diagnose STT (Table 3). Genetic material of rickettsia was found in 53 (63.8%) inoculation eschar swabs. *R. sibirica* DNA was detected in 49 (59.0%) and *R. heilongjiangensis* DNA was detected in 4 (4.8%) tested samples. The positive and negative results of the 83 patients'swabs analysis using two assay kits coincided.

Table 3: Study of swabs collected from eschars for patients from Gorno-Altaisk using two assay kits

Number of Tested Samples	Number of Positive DNA Detections (%) Using the Assay Kit				
	"RealLine *Rickettsia species"*		**"RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis*"**		
	Positive	Negative	*R. sibirica*	*R. heilongjiangensis*	Negative
83	53 (63.9)	30 (36.1)	49 (59.0)	4 (4.8)	30 (36.1)

The negative results of real-time PCR obtained upon examination of 30 patients could be caused by an extremely low concentration of rickettsia in eschars, which, after the appearance of a skin reaction, gradually decreases because of pathogens relocating to deeper skin layers, as well as their degradation by the immune system of the infected person [35]. Moreover, the development of the eschar could have been possibly caused by different infections [18, 19].

It should be noted that far from all patients having a TBR associated with *R. sibirica* and *R. heilongjiangensis* develop the eschar. For this reason, for a more efficient detection of rickettsia DNA, L.S. Karan et al. used various clinical samples from their patients [5]. We employed a similar approach to examine the patients who were preliminary diagnosed with the Siberian tick typhus on the basis of their epidemic anamnesis and

demonstrated symptoms. As a result of testing four types of clinical material using the "RealLine *Rickettsia species*" assay kit, the diagnosis was confirmed for 54 out of 71 (76.1%) patients (Table 4). Rickettsia DNA was found in 96.4% of eschar swabs (27 out of 28 samples) and in 12 out of 14 (85.7%) eschar biopsies. In the examined patients, the inoculation eschar developed due to ticks bite was registered only in 30 (42.3%) cases. Therefore, the determination of rickettsia genetic material in clinical samples of another type was of special interest.

The testing of LBF samples using PCR identified the presence of *Rickettsia species* DNA in 39 out of 67 (58.2%) patients; 16 of them also had positive results for eschar swabs analysis, 9 had negative eschar swabs, and 14 patients did not have eschar swabs available.

Table 4: Testing of various clinical samples from 71 patients with a preliminary tick-borne rickettsiosis diagnosis using the "RealLine Rickettsia species" assay kit

Results of Detection of the *Rickettsia Species* DNA in Clinical Samples from Patients				**Number of Patients with Real-Time PCR Results**	
LBF	**PA Biopsy**	**A swab collected from the inoculation eschar**	**Urine**	**Positive**	**Negative**
+	+	+	+	2	
+	+	+	-	2	
+	-	+	-	1	
-	+	+	-	2	
-	+	+	+	1	
+	+	+	N.D.	3	
-	+	+	N.D.	1	
-	+	N.D.	+	1	
-	N.D.	+	+	1	
-	N.D.	+	+	1	
-	N.D.	+	-	2	
+	N.D.	+	N.D.	8	
-	N.D.	+	N.D.	1	
+	N.D.	N.D.	+	8	
+	N.D.	N.D.	-	2	
-	N.D.	N.D.	+	2	
-	N.D.	N.D.	-		7
N.D.	N.D.	+	+	1	
N.D.	N.D.	+	-	1	
N.D.	-	N.D.	-		1
N.D.	N.D.	-	N.D.		1
+	N.D.	N.D.	N.D.	14	
-	N.D.	N.D.	N.D.		8
			Total	54 (76.1 %)	17 (23.9 %)

Thus, the LBF testing allowed confirming the preliminary Siberian tick typhus diagnosis additionally for 24 patients. Moreover, in the course of the complete examination of the LBF samples for the presence of molecular markers of other TBI pathogens, *B. miyamotoi* DNA was found in two persons, suggesting that the fever in the patients was caused by borrelia rather than rickettsia infection. This provides another confirmation of the need for a comprehensive laboratory study of such patients to detect a series of TBI pathogens.

In case of urine samples analysis (34 patients), the presence of genetic material of rickettsia was determined in 17 (50.0%) cases with low concentration (Ct > 35). Note that urine was the only type of clinical material, which study confirmed the preliminary diagnosis for two patients. For other 15 patients, *Rickettsia species* DNA was found in at least one out of three other types of biosamples.

Upon sequencing rickettsia DNA contained in the swabs collected from the eschar and in LBF of 37 patients for two genes, *gltA* and *ompA*, we found that they were infected with the pathogenic *R. heilongjiangensis*. All determined nucleotide sequences were completely homologous to each other, as well as to the sequences of these genes in the isolates of rickettsia of this species (*AY280709* and *AH012829*), isolated in Khabarovsky kray earlier [14].

Thus, the performed study demonstrated that the results of PCR detection of rickettsia DNA in biopsies and eschar swabs corresponded exactly, which confirmed the earlier published data on the efficiency of using swabs for TBR diagnostics. To confirm the diagnosis in patients without eschar, the samples of LBF can be used for PCR. We studied the possibility of using samples of whole blood and plasma from the patients to extract total DNA and analyse it for the presence of genetic material of rickettsia. The study included 53 patients with suspected TBI. When testing the eschar swabs and LBF, *Rickettsia spp.* DNA was detected in 37 out of 53 (69.8 %) patients (Table 5). In the plasma tests, genetic material of rickettsia was found in 15 out of 37 (40.5 %) patients, and when testing the whole blood, only in 3 (8.1%) patients, probably due to low concentration of rickettsia in this clinical material. An increase in the blood volume used for extraction of total DNA from 50 to 100 µl resulted in amplification inhibition. Erythrocytes are known to play an important role in this process; their fraction as well as their components account for nearly half of the whole blood volume, [36].

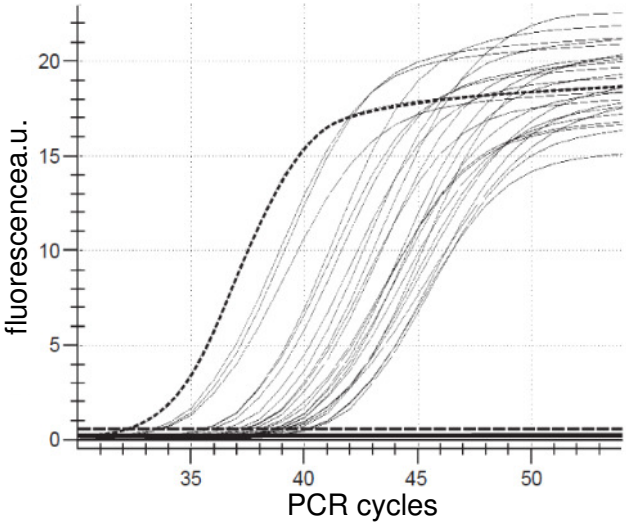
Table 5: Study of clinical material from 53 patients with the suspected TBI using the "RealLine *Rickettsia species*" assay kit

Type of Clinical Sample (Volume Used for Extraction of DNA, µl)	Number of Samples	
	Tested	Containing *Rickettsia spp.* DNA (%)
Swab collected from the eschar (100)	26	22 (84.6)
LBF (100)	53	23 (43.4)
Blood plasma (100)	52	15 (28.8)
Whole blood (50)*	50	3 (6.0)

* Increase in the volume of the whole blood for extraction of total DNA leads to amplification inhibition.

In order to remove the inhibitors, the "RealLine Hemolytic" assay kit was developed, intended for lysing erythrocyte cell membrane and obtaining the cell pellet used for extraction of total DNA. We used this assay kit for preliminary treating the samples of whole blood with a volume of 250 µl from 57 patients with the STT diagnosis. In the real-time PCR of total DNA obtained from these samples, genetic material of *Rickettsia spp.*was detected in 32 (56.1%) patients (See Fig.).

Figure: Diagram of changes over the course of the study in fluorescence of total DNA extracted from 250 µl of whole blood of 32 patients using the "RealLine DNA *Rickettsia species*" assay kit with preliminary treatment by hemolytic substance.



Thus, the study of a large number of ticks collected in different regions of Russia has shown that the "RealLine *Rickettsia species"* assay kit detects DNA of 11 different species of rickettsia, including 7 pathogenic species capable of causing human diseases. The "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis"* multiplex kit can simultaneously detect genetic material of two pathogenic rickettsia, which are the pathogens of Siberian tick typhus, prevailing in Siberia and the Far East.

It was also shown that both of new kits can be successfully used in epidemiologic studies to determine the circulation of pathogenic rickettsia in their natural foci, detect their DNA in ticks whose bite led to consequences, as well as to analyse clinical samples in the course of laboratory diagnostics of rickettsioses.

In our opinion, when working with clinical material of the examined patients, it would be more expedient to use the "RealLine Rickettsia species*"* assay kit, intended for detection of a conservative region in gene *gltA* (citrate synthase) found in all rickettsia. These include not only pathogenic, but also opportunistic pathogenic rickettsia *R. raoultii* and *R. helvetica*, which are associated with human infection in secondary immunodeficiencies [7, 37, 38]. Furthermore, the detection of genetic material of *Candidatus rickettsia tarasevichiae* is possible, whose association with the morbidity in the RF has earlier been considered by a group of Russian researchers from Omsk [39] and was recently established in China for several dozens of people [40].

The most suitable biosamples for extraction of total DNA for the assays using the developed kits for rickettsiosis detection are eschar swabs, leucocyte blood fraction and whole blood, the latter requiring a preliminary treatment with the "RealLine Hemolytic" assay kit.

The assay kits for the real-time PCR detection of genetic material of TBI pathogens: "RealLine TBEV", "RealLine *Borrelia burgdorferi s.l."*, "RealLine *Anaplasma phagocytophilum/Ehrlichia muris*, *EhrlichiaChaffeensis"*, "RealLine *Borrelia miyamotoi"*, "RealLine *Babesia species*", "RealLine *Rickettsia species"*, and "RealLine *Rickettsia sibirica/Rickettsia heilongjiangensis*"are affordable for laboratories, and their application can significantly improve the differential diagnostics of the diseases, especially when the patient does not display any pathognomic symptoms [31, 41–43].

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- **Anaplasma phagocytophilum**
- **Ehrlichia chaffeensis**
- **Ehrlichia muris**
- **Rickettsia sibirica**
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