SCIENTIFIC REPORT

RealLine *Borrelia burgdorferi s. l.*

A New Assay Kit for Detection of Tick Infection with Human Pathogenic *Borrelia* by Real-Time Polymerase Chain Reaction

Ixodic tick-borne borreliosis (ITB), or Lyme disease, is a transmissible, infectious disease that is among the most widely spread zoonotic diseases. The disease is characterised by a variety of clinical implications affecting numerous organs and systems (skin, heart, blood vessels, pancreas, liver, nervous system, musculoskeletal system, etc.). It proceeds often in a latent form, with a tendency to relapse, chronic course, stretched over months to years, often resulting in extended disability or invalidity of the patient.

The ITB is caused by *Borrelia* species belonging to *Borrelia burgdorferi sensu lato* (s. l.) complex of 12 genospecies [1]. Currently, pathogenicity for humans has been confirmed for three *Borrelia* genospecies of these spirochetes: *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto (s. S.) [2]. *Borrelia* species carriers and transmitters are ticks of *Ixodes* genus. In Russia, *I. persulcatus* and *I. ricinus* are of major epidemic importance. [3, 4]. Contamination of ticks with *Borrelia* in various biotopes and regions of the Eastern Europe is 10 to 60% [3]; higher rates are observed in the North-Western regions of Russia [5].

ITB pathogens enter the human body with the saliva of infected adult ticks, their larvae, and nymphs during bloodsucking. It is also possible to transmit *Borrelia* if the tick is removed from pets and is accidentally crushed resulting in the contact of the contents of ticks intestine or faeces with microtrauma of the skin or on conjunctiva of the eye.

Recently, city residents are about 70 to 80 % of ITB patients, which is associated to both with an increase in visiting natural foci of tick infections and an occurence of urban populations of ticks [6].

According to several reports, every year brings increasing number of patients who are seeking assistance in healthcare facilities after a tick bite. As an example, in Russia earlier 232 to 379 thousands victims of bites were annually registered, then in only 8 months of 2009, 482,600 people turned to healthcare facilities on this issue [7].

In Russia, the *Borrelia* habitat extends from the Baltic Sea to the Pacific Ocean, and from 6.3 to 8.7 thousand cases of ITB are registered annually. In 2008, the incidence rate of tick-borne Borreliosis in the country was 5.42 cases per 100 thousand people; however, it was 8 times higher in the Tomsk region, 7 times higher in the Kirov region, and 5 times higher in the Yaroslavl and the Vologda regions. Apperently the official data do not reflect the actual picture of ITB expansion in Russia. This is related on the one hand to an insufficient diagnostics of Borreliosis infection and on the other hand to the fact that only about 10% of persons suffering from a tick bite pass examination. Many of them do not notice parasite crawling or sucking, forget about the contact, or do not have an opportunity to get an urgent analysis of the tick for the presence of pathogens and the conduct of a preventive therapy [8, 9]. There is reason to assume that the real incidence of ICB in the Russian Federation does not differ from countries located in similar geographic and climatic conditions. In Slovenia, Austria, Germany, Sweden, and Finland, the rate of ITB infections is about 50–130 cases per 100,000 persons [10–12], which is an order of magnitude higher than Russian statistics, while tick infestation with pathogens of ICB is comparable, and in many cases lower than registered in Russia.

Common ITB diagnostics is based on epidemiological anamnesis (tick sucking, living in or visiting an ITB endemic region, visiting forest parks, including within city area), and also according clinical signs of the disease. Main clinical manifestations are: erythema migrans (ME), Buchwald atrophy (in case of skin manifestations), and Bannwarth syndrome (for neurological manifestations). Migratory erythema is a pathognomonic symptom for an early stage of ITB; however, it is observed for only 25 – 70 % of patients with the confirmed diagnosis.

The ITB diagnosis is inferred from both direct and serological methods of laboratory studies. However, many direct methods for ITB diagnostics, including direct detection of the pathogen and its antigens, are still ineffective [13–15], while serological tests do not show the required sensitivity [16].

Polymerase chain reaction (PCR) is a direct method for identifying Borrelia that is applied nowadays in developed countries more often than microscopic and cultural techniques [17, 18]. The Real-Time PCR, a modern modification of this method, is characterized by high manufacturability and reliability, and the ability to quantify the pathogen in the material under study, simplifying the analysis and interpretation of its results. The diagnostic sensitivity for PCR detection of a *Borrelia* DNA in clinical samples like blood, cerebrospinal and synovial liquids, skin biopsy, and urine, generally exceeds the sensitivity of culture methods and microscopy, but can vary substantially depending on the method used, tissue type, disease period, and its progression. At the same time, the use of PCR allows the detection of *B. burgdorferi* DNA (or RNA) in ticks with the sensitivity of more than 95 %.

The problem of early ITB diagnostics, as well as its possible long-term manifestations (10 and more years after the infection), necessitate the study of ticks taken from people for the presence of *B. burgdorferi s. l.*. Firstly, a *Borrelia*-positive result for sucking tick indicates a greatly increased likelihood of ITB development in the affected person, and may serve as a basis for the appointment of an emergency etiotropic treatment. It has been shown that a preventive antibiotic therapy in the first 5 days after the tick bite prevents disease development in 95 % of cases [14, 19]. At a later date, the efficiency is substantially lower. Secondly, the patient's history of the attack of a tick infected with *Borrelia* can help to make a correct diagnosis and the relevant treatment of the disease if a patient seeks medical assistance, even after a long period of time.

Therefore, the study of ticks that have attacked a person in order to detect a potential infection with pathogenic *Borrelia* is a required laboratory test technique that should be routinely available in healthcare facilities.

The objective of this work is to study the main analytical characteristics of the RealLine *Borrelia burgdorferi s. l.* kit and evaluation of the efficiency in analysing ticks and detecting persons infected with *Borrelia* in clinical diagnostic laboratories.

Materials and Methods: The analytical sensitivity and specificity of the "RealLine *Borrelia burgdorferi s. l.*" assay kit was determined using 11 reference strains of two genospecies, *Borrelia afzelii* (7-02, 34-01, 36-02, 43-02) and *Borrelia garinii* (3-03, 10-03, 18-05, 31-01, 51-02, 52-02, BgVir-1), in the form of *in vitro* generated normalised cultures with a concentration of 1.05 –2.55 x 10^7 cell/ml (Virion, Tomsk, Russia). *Borrelia burgdorferi s. s.* genospecies were represented by B-31 strain, and *Treponema pallidum* spirochetes were represented by *Nichols* strain with a concentration of 1.5 x 10^7 cell/ml. Serial dilutions were prepared from all cultures listed above strains with borrelia content from 500 to 10^7 cell/ml and treponeme content from 1.5 x 10^5 to 1.5 x 10^7 cell/ml. Using the "RealLine Extraction 100" kit (BIORON Diagnostics, Germany), total nucleic acids (NA) were extracted from each dilution and then analysed by Real-Time PCR.

The study used 543 individual *Ixodes* ticks collected by flagging [20] 40 km from Novosibirsk, Russia in May 2009. Each of the caught ticks was placed in an individual plastic microtube and frozen at -70 °C.

Total NA Extraction from Ticks: 96 % Ethanol (300 µl) was added to a tube with a tick and the tube was vortexed for 10–15 sec (Bio Vortex). Tubes were spinned briefly to collect any drops, and alcohol was removed with a pipette having a disposable tip, or with a vacuum aspirator. The ticks in the tubes were washed with 500 µl of 0.15 M NaCl as described above, and then were frozen for 5 min in liquid nitrogen (or for 20 min in a freezer at -70 °C). The frozen tick was thoroughly ground in the tube with an individual sterile metal pestle and suspended in 250 µl of TE buffer (pH = 8.0). After stirring the tube (Bio Vortex, 10–15 sec), the particles of chitinous plates were precipitated by brief centrifugation to obtain a supernatant (suspension of tick organs and soft tissues). A fraction of total NA was extracted from 100 µl of the supernatant as described above (the remainder was frozen and stored at -70 °C). The volume of the extracted total NA solution was 200 µl.

Detection of *Borrelia* DNA in Ticks: The detection was performed on iQ5 iCycler, a thermocycler with Real-Time fluorescence detection (Bio-Rad, USA), using "RealLine *Borrelia burgdorferi s. l"* assay kit. The study was performed with 30 µl of the total NA solution extracted from each of 543 ticks (the remainder was frozen and stored at -70 °C).

Detection of Genotypes of *Borrelia* circulating in Ticks: The study was performed with 57 out of 314 ticks, in which a genetic material of ITB pathogens had been detected using "RealLine *Borrelia burgdorferi s. l*" kit. The samples of total NA obtained from the 57 ticks were used to produce 480 bp DNA fragment of the *rec A* gene of *Borrelia*. The fragments were Sanger sequenced on both DNA strands using BigDye terminator sequencing kit and ABI Prism 3100 DNA Analyser (Applied Biosystems, USA). Resulting nucleotide sequences were processed and compared to the corresponding data from the GenBank database with the BLAST search system (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Vector NTI v 8.0 software [21]. The latter was also used to compare the nucleotide sequences for *rec A* genes in *Borrelia* of three genospecies and *Treponema pallidum*.

Results and Discussion: The "RealLine *Borrelia burgdorferi s. l."* assay kit has been developed in compliance with the general principles for designing of the "RealLine kits" [22].

For hybridisation of primers and probes, a 95 bp conservative region of the *rec A* gene contained in the linear chromosome of *Borrelia* was chosen, which has substantial differences from the corresponding region of the *rec A* gene in *Treponema pallidum*, the syphilis pathogen.

In certain assay kits of similar intended use, the ribosomal RNA fragment serves as the amplification target. Here, a DNA fragment was chosen as a target, eliminating the need to perform the reverse transcription step, which simplifies the analysis, shortens its duration, and reduces the risk of contamination. Furthermore, compared to DNA, RNA molecules are more prone to destruction upon storage and transportation of both clinical samples and ticks.

The "RealLine *Borrelia burgdorferi s. l."* assay kit includes an internal control sample (IC), which is added to all test samples and passes all analysis steps together with them, including DNA extraction. Addition of IC helps to track any losses of nucleic acids in the course of sample preparation, control the entire PCR process, including detection of a possible reduction in amplification efficiency if the test sample contains any Taq-polymerase inhibitors.

A positive advantage of Real-Time PCR is the possibility to determine the initial number of copies of the analysed DNA in the test sample. The intensity of the fluorescence signal is detected by the device during the course of Real-Time PCR is proportional to the concentration of the amplification product in the reaction mix at the moment of measurement.

In the course of Real-Time PCR, the fluorescence from each test sample starts to reliably exceed the background level only after a certain number of amplification cycles, i.e., after passing the threshold cycle (Ct). The lower the initial DNA copy number in a test sample, the greater the number of amplification cycles is required to reach the threshold value, i.e., the higher is Ct. And vice versa: the lower the Ct value for a given sample, the higher is its initial content of the target DNA [23].

The "RealLine *Borrelia burgdorferi s. l.*" kit is mainly intended for laboratory studies to detect the genetic material of ITB pathogens in various biological samples. A quantitative evaluation of the *Borrelia* content in test samples using the kit requires additional positive control samples (calibrators) with a known concentration of the target DNA. In the study to determine the analytical sensitivity of the "RealLine *Borrelia burgdorferi s. l.*" assay kit we employed the samples of a total NA extracted from serial dilutions of 12 reference strains of *B. afzelii*, *B. garinii*, and *B. burgdorferi s. s.* with a known concentration of these microorganisms as such calibrators. The 30 µl samples used in the PCR analysis contained from 50 to 10^6 copies of the target DNA (*rec A gene*).

The results of the experiments demonstrated that Real-Time PCR performed using the "RealLine *Borrelia burgdorferi* s. l." kit detects all 12 strains of three *Borrelia* genospecies. The analytic sensitivity of the kit at the 95 % confidence level ($p < 0.05$) is 50 genome equivalents (g/e) of the *B. burgdorferi s. l.* DNA in the test

sample. The Ct value showed a linear dependence on the quantity of the initial DNA in the samples used for PCR in the range from 50 to 10^6 g/e of DNA.

To assess the specificity of the "RealLine DNA *Borrelia burgdorferi s. l.*" kit, we studied the samples of a total DNA extracted from serial dilutions of *Treponema pallidum* Nichols strain and containing from 1.5 x 10^4 to 1.5 x 10^6 copies of the corresponding *rec A* gene in the test sample. A negative result for all these samples confirmed that specific primers and probes were chosen correctly, even though the degree of homology of the complete nucleotide sequences for this gene in the ITB and syphilis pathogens is 60%.

To assess the compatibility of results of *Borrelia* DNA detection by the developed kit and the kit with similar intended use, samples of total NA extracted from 80 ticks were analysed in parallel by the "RealLine *Borrelia burgdorferi s. l.*" and "Borrelia burgdorferi" (DNA Technology LLC, Moscow) assay kits.

Table 1

Results of *Borrelia* DNA Detection in 80 ticks using the "RealLine DNA *Borrelia burgdorferi s. l.*" assay kit and the "*Borrelia burgdorferi*" assay kit

Assay Kit	Number of Samples Registered as		Number of Coincided Samples		
	Positive	Negative	Positive	Negative	Total
RealLine *Borrelia burgdorferi s. l.*	56	24	48	24	72 (90%)
Borrelia burgdorferi	48	32			

The obtained results coincided in 90% cases.

"AmpliSensR *Borrelia burgdorferi* sensu lato-FL" assay kit (InterLabService Ltd., Moscow) was used as a reference kit as well. Using this assay kit, the genetic material of three species of *Borrelia* was detected using reverse transcription PCR, with a fragment of 16S RNA as an amplification target. In this case, the coincidence rate for the results from two kits was 93.4% (Table 2).

Table 2

Results of *Borrelia* Genetic Material Detection in 76 Ticks Using "RealLine DNA *Borrelia burgdorferi s. l.*" and "AmpliSens® *Borrelia burgdorferi sensu lato*-FL" assay kits

Assay Kit	Number of Samples Registered as		Number of Coincided Samples		
	Positive	Negative	Positive	Negative	Total
RealLine *Borrelia burgdorferi s. l.*	45	31	40	31	71 (93.4%)
AmpliSens® *Borrelia burgdorferi sensu lato*-FL	40	36			

It should be noted that discrepancies in the results of tests performed with the compared kits were found only for samples with a very low concentration of *Borrelia* NA, which had the values of Ct ≥ 37 in the PCR run with the "RealLine *Borrelia burgdorferi s. l.*" assay kit. The negative test result for these samples obtained with two reference kits may be due to differences in their diagnostic sensitivity.

To assess the efficiency of detecting ITB pathogens in their natural carriers using the "RealLine *Borrelia burgdorferi s. l.*" kit, we studied the total NA extracted from 543 tick individuals collected in the suburbs of Novosibirsk, Russia. As a result of PCR, DNA of human pathogenic *Borrelia* was found in 314 ticks (57.8 %). Such a high infection rate does not exceed the upper boundary for the range of infection rates of Ixodes ticks in the Novosibirsk region (15.0 – 58.3 %) published earlier [24].

One of the factors for ITB development is the number of pathogenic *Borrelia* entering a human body when an infected tick feeds. The infecting dose, apparently, has a strong dependence on the number of ITB pathogens circulating in a given tick. We performed a quantitative evaluation of human pathogenic *Borrelia* contained in 314 ticks with the positive PCR outcome. To this end, we used Ct values in the range of 26–40 amplification cycles. Depending on the selected range of Ct values, all test samples were divided into 4 groups, with each group corresponding to a certain level of initial target DNA concentration (Table 3).

Table 3

Evaluation of the Number of Individuals of Human Pathogenic *Borrelia* in 314 Ticks Using Real-Time PCR Ct Values

Indicators	Group			
	1	2	3	4
Ct range obtained during PCR for 314 samples	26–28	29–31	32–34	35–40
Number of DNA copies in the test sample corresponding to the Ct range	8,000–2,000	1,000–250	100–50	< 50
Estimated content of *Borrelia* individuals in a single tick	1.4×10^5 to 3.4×10^4	1.7×10^4 to 4.2×10^3	2×10^3 to 8×10^2	< 800
Number of ticks with the given *Borrelia* content	42	72	64	136
Fraction of ticks with the given *Borrelia* content, %	13.4	22.9	20.4	43.3

The analysis of the obtained results demonstrates that, from ticks collected in the suburbs of Novosibirsk, the fraction of individuals with a high content of ITB pathogens (1.4×10^5 to 4.2×10^3 *Borrelia* cells) is 36.3 %, and the fraction with a low content (less than 800 cells) is 43.3 %. The maximal number of *Borrelia* circulating in a single tick individual reached 140 thousand, which agreed with the data of other researchers on the presence of up to 200 – 220 thousand of these spirochetes in ticks [25, 26].

It should be noted that the probability of ITB development in humans after being bitten by a tick infected with *Borrelia burgdorferi s. l.* is rather high. In the North-Western region of Russia, a manifestation of the disease with acute or subacute ITB progression was observed in 20 – 25 % of patients who were bitten by an infected tick [19]. In another paper, the case of 72 residents of the St. Petersburg region who were bitten by ticks with *Borrelia* but who did not receive a preventive therapy, is described. Among them, 16 patients became infected with ITB (22.2 %) without regard for other tick-borne infections [27]. Therefore, patients who were attacked by a tick with detected human pathogenic *Borrelia*, irrespective of their quantitative content, must undergo a timely preventive etiotropic treatment, preferably in a specialised healthcare facility.

By now, several scientific labs are working on the prevalence of different genospecies of *Borrelia burgdorferi s. l.* in the regions of the Russian Federation [28, 29]. To assess the degree of incidence of specific genospecies of ITB pathogens in the studied tick sampling, we randomly selected 57 out of 314 tick individuals that were found positive by Real-Time PCR. The samples of total NA obtained from 57 ticks were used for amplification, extraction, and subsequent sequencing of a 480 bp DNA fragment for the *rec A* gene, whose nucleotide sequences in different species of the *Borrelia* genus have characteristic differences. The obtained nucleotide sequences were compared to data represented in the GenBank database with BLAST search system. As a result of the phylogenetic analysis, the DNA for *Borrelia* circulating in 39 out of 57 (68.4 %) of the tested ticks was attributed to *B. garinii genospecies*, for 18 ticks (31.6%) to *B. afzelii*, with no other *Borrelia* species found. The prevalence of *B. garinii* among ITB pathogens available in the Novosibirsk region was noted earlier by other authors [30].

Summing up, based on the results of the performed studies it was established that the new "RealLine *Borrelia burgdorferi s. l.*" kit based on PCR with Real-Time detection of the amplification products **ensures a reliable detection of DNA for ITB pathogens of three genospecies: *B. afzelii*, *B. garinii,* or *B. burgdorferi s. s.*, with analytical sensitivity of 50 DNA copies in the test sample**. It was demonstrated that the kit can be used in the laboratories of healthcare facilities as the routine express assay for detection of pathogenic *Borrelia* in the ticks and prevention of ITB development in the patients.

The studies performed with the "RealLine *Borrelia burgdorferi s. l."* kit have shown that 57.8 % (314 out of 543) of ticks collected in the suburbs of Novosibirsk were infected with *B. garinii* or *B. afzelii*, which amount ranges from 1.4 x 10^5 to 4.2 x 10^3 *Borrelia* in 36.3% of ticks.

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These results are represented by

BIORON Diagnostics GmbH

Rev01_1118

Portfolio for Detection Kits for Tick-associated diseases:

- **Borrelia burgdorferi s.l. complex:**
  B. afzelii, B. garinii, B. burgdorferi sensu strictu, B. spielmannii, B. bavariensis, B. bisettii, B. lusitania, B. valaisiana
- **Borrelia miyamotoi**
- **Anaplasma phagocytophilum**
- **Ehrlichia chaffeensis**
- **Ehrlichia muris**
- **Rickettsia sibirica**
- **Rickettsia heilongjiangensis**
- **Babesia species:**
  B. microti, B. venatorum, B. divergens, B.duncani, B. canisis
- **Tick Borne Encephalitis Virus**

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