Original research article UDK 595.42:[616.98:579.834
https://doi.org/10.46784/eavm.v14i1.269

# DETECTION OF *BORRELIA* SPIROCHETES IN TICKS USING q16 REAL-TIME PCR

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## Abstract

Lyme disease is a tick-borne disease caused by *Borrelia burgdorferi* sensu lato spirochaetes. It is transmitted by several hard ticks of the *Ixodes* genus, mainly *Ixodes ricinus* in Europe. Higher temperatures caused by climate changes are linked to a heightened activity of ticks for most of the year. Therefore, the awareness of tick-borne diseases is increasing in the region. The aim of this study was to estimate the use of molecular method with Genesig q16 PCR in real time, (Primerdesign Ltd., United Kingdom), as a diagnostic tool for rapid identification of causative agent for Lyme disease in ticks. *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* are the most important causative agents of Lyme disease in this region. With this method a targeted gene is detected and it has been previously discovered that it is a good genetic marker for the three strains of *Borrelia*. A total of 90 ticks were collected after being removed from humans. Every tick collected was identified regarding its species. A total of 79 ticks belonging to the *Ixodes* genus were tested for the presence of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* using real-time PCR assay targeting the recA gene. In total, 8 of them tested positive. Representative samples were tested by conventional PCR and the obtained results were in accordance with the ones obtained by qPCR. This study showed that the Genesig q16 Real-Time PCR is an easy diagnostic test for fast detection of *Borrelia* spirochetes in ticks.

**Key words:** Lyme disease, tick-borne disease, real-time PCR, *Borrelia*.

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# UPOTREBA q16 REAL-TIME PCR-a ZA UTVRĐIVANJE PRISUSTVA SPIROHETA *BORRELIA* U KRPELJIMA

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## Kratak sadržaj

Lajmsku bolest uzrokuju spirohete *Borrelia burgdorferi s.l*, a prenose krpelji. Nosioci spiroheta su uglavnom tvrdi krpelji iz roda *Ixodes*, i to najčešće *Ixodes ricinus* u Evropi. Klimatske promene i porast spoljašnjih temperature uslovili su i povećanu aktivnost krpelja tokom većeg dela godine, pa se samim tim u regionu povećao značaj bolesti koje se prenose krpeljima. Cilj ove studije bio je da se proceni upotreba molekularne metode pomoću Genesig q16 PCR-a u realnom vremenu (Primerdesign Ltd., Velika Britanija), kao dijagnostičkog alata za brzo otkrivanje uzročnika lajmske bolesti kod krpelja u rutinskoj laboratorijskoj dijagnostici. *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* i *Borrelia afzelii* su najznačajniji uzročnici lajmske bolesti u ovom regionu. Ovom metodom se otkriva ciljni gen, za koji se prethodno pokazalo da je dobar genetski marker za ove tri vrste. Uklanjanjem sa ljudi, prikupljeno je ukupno 90 krpelja. Svaki od krpelja je identifikovan u odnosu na vrstu. Ukupno 79 krpelja, za koje je utvrđeno da pripadaju rodu *Ixodes*, su testirani na prisustvo *Borrelia burgdorferi* s.s, *Borrelia garinii* i *Borrelia afzelii* pomoću PCR testa u realnom vremenu, usmerenog na gen recA. Rezultati testa su pokazali 8 pozitivnih uzoraka. Analiza reprezentativnih uzoraka drugom metodologijom je dala usaglašene rezultate. Ova studija je pokazala da je upotreba Genesig q16 PCR u realnom vremenu jednostavan test za brzo otkrivanje spiroheta Borrelia kod krpelja.

**Ključne reči**: Lajmska bolest, bolesti prenosive krpeljima, PCR u realnom vremenu, *Borrelia*

## INTRODUCTION

Lyme disease is a tick-borne zoonosis, caused by *Borrelia burgdorferi* sensu lato (s.l.) bacteria. Lyme disease mostly occurs in people and dogs, but it affects other animals as well (Potkonjak et al., 2016b). In humans, vector-borne diseases represent more than 17% of all known infectious diseases (WHO, 2017). Since the discovery of the cause of Lyme disease (Steere et al., 1977; Burgdorfer et al., 1982), tick-borne infections are the subject of intensive research all over the world. Lyme disease is the most prevalent arthropod-borne infection in the Northern Hemisphere (Stanek et al. 2012). Hard ticks, mostly belonging to *Ixodes* genus are the main vectors for *Borrelia* spirochetes. *Ixodes ricinus* is a predominant vector of Lyme disease in Europe. Ticks that carry *B. burgdorferi* s.l. in Serbia belong to *I. ricinus* species as well (Savić et al., 2010).

The change of vegetation and current climate changes (particularly mild winters) increase the activity of the tick population. Current climate changes affect regional vector introduction, vector shift to higher latitudes and altitudes and extended annual periods of vector activities (Leschnik, 2020). There is no longer seasonal occurrence of ticks. So far we know that Serbia is an endemic area for a large number of tick-borne infections (Potkonjak et al., 2016a).

Diagnosis of *B. burgdoferi* s.l. in ticks relies on the detection of bacteria in their abdominal gut. In live ticks, detection of spirochetes is possible by dark-field microscopy. Detection of bacteria in dead ticks is not possible when this method is used. Compared to the previous assay, molecular method, such as PCR assay, has higher sensitivity for the detection of causing agent and improves the diagnostics in the laboratory. It can be performed on both live and dead ticks, which is a considerable advantage. With the progress in molecular diagnostics, new species, strains, and genetic variants of microorganisms are detected in ticks worldwide, and the list of potential tick-borne pathogens is increasing (Dantas-Torres et al., 2015). In the past decade, most of the research conducted in Serbia widely used molecular methods for proving the presence of pathogens in ticks (Milutinović et al., 2008; Savić et al., 2010; Tomanović et al., 2010a; Tomanović et al., 2010b; Radulović et al., 2011; Tomanović et al., 2013; Potkonjak et al., 2016a; Potkonjak et al., 2016b; Potkonjak et al., 2017). The screening of DNA samples was performed mostly by conventional PCR assay, targeting specific bp fragment. Five *Borrelia* species are confirmed as pathogens in Europe (*B. burgdorferi* sensu stricto (s.s.), *B. afzeli*, *B. garinii*, *B. spielmani* and *B. bavariensis*). In general, the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks from Serbia is 21.1% - 42.5% (Milutinović et al., 2008; Čekanac et al., 2010; Savić et al., 2010; Potkonjak et al., 2016a; Potkonjak et al., 2016b). So far, the following species from ticks have been registered in

Serbia: *B. lusitaniae*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. burgdorferi* s.s. and *B. bavariensis* (Ćakić et al., 2019); *B. burgdorferi* s.s, *B. garinii* and *B. afzelii* are the most significant causative agents of Lyme disease in this region (Milutinović et al. 2008; Savić et al, 2010; Potkonjak et al. 2016).

This study aims to evaluate the use of Genesig q16 Real-Time PCR (designed and launched by Primerdesign Ltd. UK) as a tool for detection of causative agents of Lyme disease in ticks in routine laboratory conditions.

## MATERIAL AND METHODS

### *Sample collection*

The ticks were collected from human patients who visited their general practitioner because of a tick bite. A total of 90 ticks were collected. The species of each tick was identified at the Scientific Veterinary Institute "Novi Sad". Seventy-nine ticks were identified as *I. ricinus*. These ticks were analysed for the presence of *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* using q16 real-time PCR method. The collected ticks were stored at -20 °C before the analysis. Before the extraction of DNA, a two-step wash protocol of ticks was performed (using 70% ethanol and distilled water). After that, the samples were processed in TissueLyser LT (Qiagen). Sample disruption with small beads and phosphate buffered saline (PBS) is a crucial step before extraction. This way the bacteria are released from hard tick and the isolation of potential DNA is then possible.

### *DNA extraction*

DNA was isolated from whole tick tissues using the Genesig Easy extraction kit (Primedesign Ltd), by adding metal beads and using a magnetic separator. The DNA extraction is a six-step process that takes 45 min to complete. Sample lyses are stimulated by incubation with lysis buffer and proteinase K. Proteinase K given in the extraction kit is active at room temperature, and when using the incubation it is shorter compared to other protocols of DNA extraction. This step is followed by the addition of binding buffer and Genesig easy magnetic beads. After magnetic separation, the magnetic beads are washed through a three-step process with buffer 1, buffer 2 and 80% ethanol in order to remove contaminants and salts. In the last step, high purity DNA/RNA is eluted with a slightly saline elution buffer. Prepared in this way, the ticks DNA can be used directly in reactions as a sample.

The detection of the presence of a target DNA was done by real-time quantitative PCR (Genesig q16, Primedesign ltd), using Lyme disease genesig® Easy

kit. The kit is designed for the in vitro quantification of "Lyme disease genome". This kit detects a target gene (recA gene) which has previously been proved to be a good genetic marker for all three species (*B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.). The primers and probe sequences in this kit have 100% homology with over 95% of reference sequences in the NCBI databases based on comprehensive bioinformatics analysis.

There are 50 cycles in this qPCR protocol. Every cycle consists of: 2 min of enzyme activation at 95 °C, followed by 10 s of denaturation at 95 °C and 1 min of data collection at 60 °C.

### *Interpretation of the results*

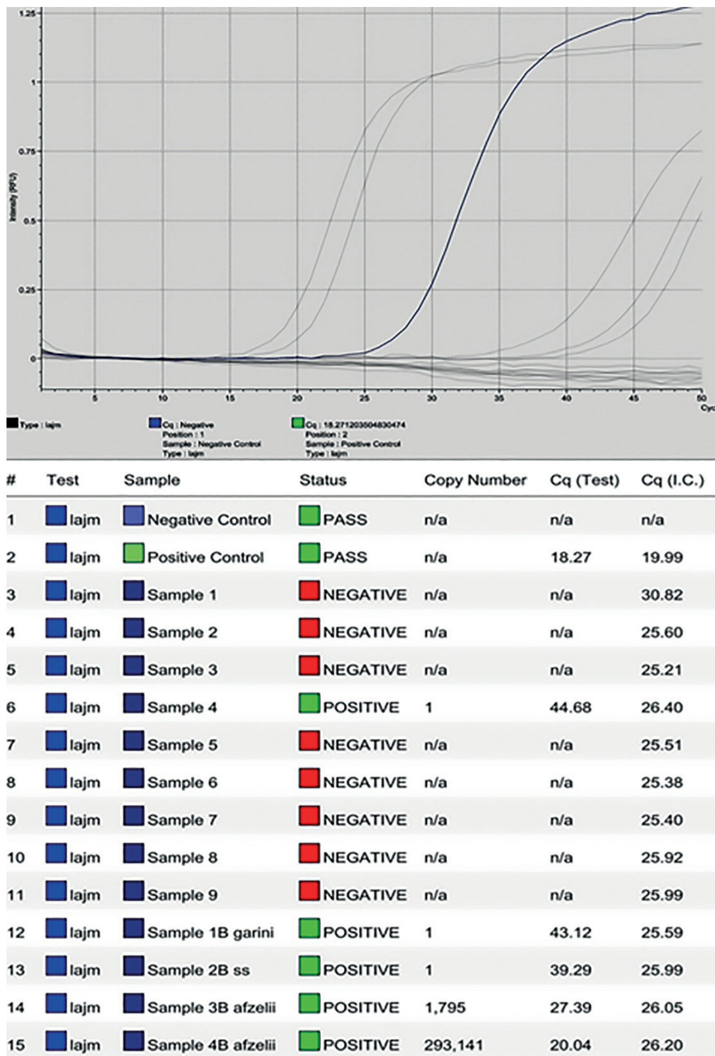
The interpretation of the results was done according to the instructions of the producer (PrimeDesign). Positive control template is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised. Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted. If the sample amplifies > 5 Cq earlier than the negative control, then the sample should be reinterpreted with the negative control verified as negative. If the sample amplifies < 5Cq earlier than the negative control, then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

### *Detection of B. burgdorferi s.l. DNAs in tick samples by conventional PCR*

Representative DNA samples prepared from tick tissue were subjected to additional method for detection of *B. burgdorferi* s.l. - *rrf-rrl* rDNA intergenic spacer PCR. Primers corresponding to the 3' end of 5S rDNA (*rrf*) (RIS1; 5'-CTG CGA GTT CGC GGG AGA-3' and RIS3; 5'-GGA GAG TAG GTT ATT GCC AGG-3') and the 5' end of 23S rDNA (*rrl*) (RIS2; 5'-TCC TAG GCA TTC ACC ATA-3' and RIS4; 5'-GAC TCT TAT TAC TTT GAC C-3') were used for first-step (RIS1 and RIS2) and nested-PCR (RIS3 and RIS4) under the previously described PCR conditions (Masuzawa et al., 1996). The quality of extracted DNA was evaluated by PCR targeted to the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes according to a previously described method (Radulović et al., 2010).

## RESULTS

The ticks were analysed for the presence of three pathogens *B. burgdorferi* s.l. species (*B. garinii, B. afzelii* and *B. burgdorferi* s.s.). Pathogen *Borellia* DNA was amplified in 8 of 79 ticks (10%).




| # | Test | Sample | Status | Copy Number | Cq (Test) | Cq (I.C.) |
|---|---|---|---|---|---|---|
| 1 | lajm | Negative Control | PASS | n/a | n/a | n/a |
| 2 | lajm | Positive Control | PASS | n/a | 18.27 | 19.99 |
| 3 | lajm | Sample 1 | NEGATIVE | n/a | n/a | 30.82 |
| 4 | lajm | Sample 2 | NEGATIVE | n/a | n/a | 25.60 |
| 5 | lajm | Sample 3 | NEGATIVE | n/a | n/a | 25.21 |
| 6 | lajm | Sample 4 | POSITIVE | 1 | 44.68 | 26.40 |
| 7 | lajm | Sample 5 | NEGATIVE | n/a | n/a | 25.51 |
| 8 | lajm | Sample 6 | NEGATIVE | n/a | n/a | 25.38 |
| 9 | lajm | Sample 7 | NEGATIVE | n/a | n/a | 25.40 |
| 10 | lajm | Sample 8 | NEGATIVE | n/a | n/a | 25.92 |
| 11 | lajm | Sample 9 | NEGATIVE | n/a | n/a | 25.99 |
| 12 | lajm | Sample 1B garini | POSITIVE | 1 | 43.12 | 25.59 |
| 13 | lajm | Sample 2B ss | POSITIVE | 1 | 39.29 | 25.99 |
| 14 | lajm | Sample 3B afzelii | POSITIVE | 1,795 | 27.39 | 26.05 |
| 15 | lajm | Sample 4B afzelii | POSITIVE | 293,141 | 20.04 | 26.20 |

Figure 1. The results obtained from a run of 14 samples and positive and negative controls with the status of the samples at the end of the run with Cq values.

Five representative samples were subjected to the additional method for *Borrelia* detection and evaluation of the quality of extracted DNA at the Institute for Medical Research, Belgrade University, Serbia. The quality of extracted DNA was evaluated by PCR targeted to the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes (the upper part of figure 2). After that, two positive and three negative samples (representative samples) were tested with conventional nested PCR for the presence of *B burgdorferi* s.l. Both positive and negative results were in accordance with the results of qPCR, which is shown in the lower section of Figure 2.

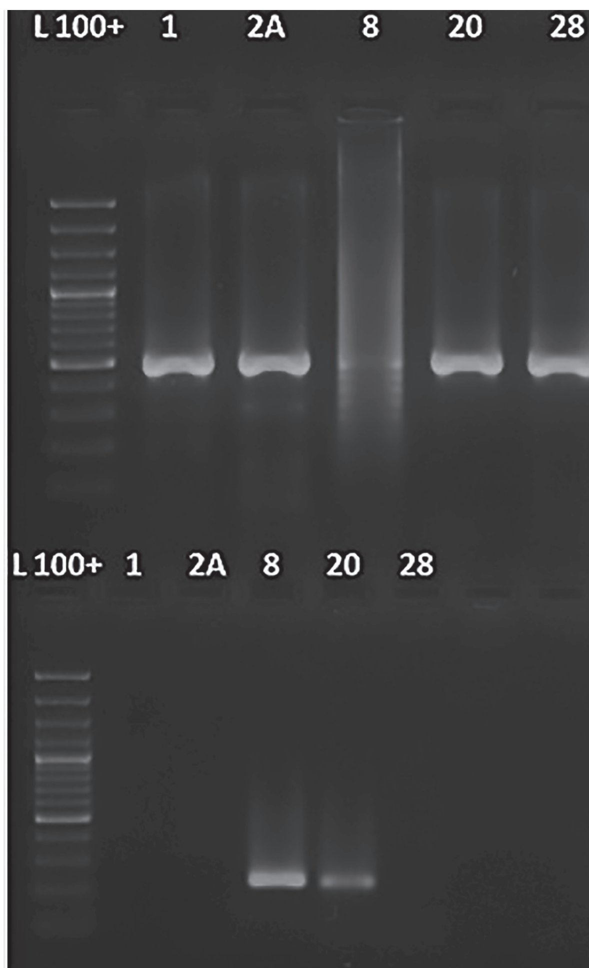



Figure 2. Conventional PCR results

## DISCUSSION

Polymerase chain reaction (PCR) technique provides more precise detection of causative agents of many infectious diseases. It is a method with high level of specificity and sensitivity, but it also has some limitations that need to be considered during the diagnostic procedure. It is very important to know what exactly is detected, in which sample and at which stage of infection. For detection of pathogen DNA, PCR method requires much less pathogen to be present in the sample than other detection methods which usually means more positive results than in the cases when other detection methods are used. Even though the sensitivity of PCR method is rather high, cross-contamination, unspecific amplification and false-negative signals are possible to occur. Another flaw of this method is that the enzymes involved in the reaction can be sensitive to inhibitors found in blood, in by-products of blood-derived DNA isolations, and in other body fluids (Zarlenga and Higgins, 2001). In the samples like engorged ticks that have taken a blood meal from a human or other animal host, determination of *B. burgdorferi* infection can be potentially inhibited (Shwartz et al., 1997) and therefore challenging. Before the extraction of DNA, removing all impurities from ticks is a very important step, as well as the proper amount of PBS for the next step /maceration: if the tick is more engorged, then the bigger amount of PBS for preparation of sample is needed. In this study, the quality of extracted DNA was estimated by internal control of Lyme disease genesig® Easy kit, and in representative samples double-checked with conventional PCR targeted to the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes following the previously described method (Radulović et al., 2010). The extracted DNA in our study was sufficient for reliable detection of Borrelial DNA.

When it comes to the detection of causative agents of Lyme borreliosis by PCR, attention also needs to be paid to test specificity (Aguero-Rosenfeld et al., 2005). The selection of gene targets for amplification has varied. PCR protocols amplified different Borrelial genes, chromosomal such as flagellin gene (Pahl et al., 1999; Ramamoorthi et al., 2005), 23S rRNA (Schwartz et al., 1992), p66 gene (Park et al., 1993), recA gene (Chan et al., 2013), and plasmids-encoded osp genes (Moter et al.,1994; Hovius et al., 2007). The most frequently cited target is the plasmid-encoded ospA, which occurs in multiple copies in each *B. burgdorferi* cell (Dumler, 2001).

Casati and coauthors (2014) successfully analysed 874 *I. ricinus* ticks, by RT-PCR method targeting recA gene. The DNA sequence analysis performed in this study enabled the characterization and identification of *B. burgdorferi*

s.l. strains. Five genospecies were detected: *B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, *B. valaisiana*, and *B. lusitaniae*. RecA gene was reliable and fast for real time-PCR detection of *B. burgdorferi* s.l. species and differentiation of three species of *Borrelia* commonly associated with Lyme disease (*B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*) in one different study as well (Mommert et al., 2001). The variability of this fragment is relatively high (23.5%) compared to other genetic markers. Only the flaA gene has shown a slightly higher variability (26.3%) than recA, but with a longer fragment sequenced (580 bp) (Casati et al., 2004).

All the other quality control criteria also were checked in this study, while performing diagnostic procedure with q16 PCR. According to the manufacturer of the kit, the quality control criteria of reaction were satisfied, which indicates that the analysis was not compromised in any way. Positive control template is expected to be amplified between Cq 16 and 23 and negative control passed as well. There were no inconclusive results. As shown in the Figure 1, internal controls of the samples and positive control were ranging between 25.21 and 26.40. The normal range of internal control according to the manufacture protocol is 25 ± 3 Cq. Anything higher than that indicates some kind of inhibition, which we did not have in our study. One sample did show internal control Cq of 30.82, but after diluting it at 1:10, the sample was re-run. After that, internal control was good, meaning that there was no inhibition.

In our study recA from *B. burgdoferi* s.l. was successfully determined. The sensitivity of PCR can be increased using a nested- PCR procedure, were two rounds of amplifications are performed (Schmidt, 1997). Representative samples in our study were re-run by nested PCR in another laboratory and the obtained results were the same as the ones gained by q16 PCR methodology.

## Conclusion

This research showed that Genesig q16 PCR real-time method is a fast and the procedure for detection of *B. burgdorferi* s.l. species with proven pathogenicity in ticks is easy to handle. Since the kit is already labelled for detection of *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s, positive results gained using this kit mean that one of these pathogen of *Borrelia* strain is present. Machine and software are easy to use. It takes a few hours to get the results (once the extraction of DNA starts). Even though it is not possible to distinguish between the *B. burgdorferi* s.l., positive results mean that there is at least one of *B. burgdorferi* s.l. pathogen strain (*B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.) present in the tick.

## ACKNOWLEDGEMENT

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia by the Contract of implementation and financing of scientific research work of NIV-NS in 2021, Contract No: 451-03-9/2021-14/200031 from 05/02/2021, and IMI-BG, Contract No: 451-03-9/2021-14/200015.

## Author's Contribution:

SS and MŽS made contributions to the idea of the publication, organisation of work and writing the manuscript; MŽS, ST and RS did the laboratory analysis, MS participated in the writing of the manuscript and ST, RS and SS reviewed the manuscript and participated in the final draft of the manuscript.

## Competing interest

The authors declare that they have no competing interest.

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Received: 22.07.2021.
Accepted: 23.08.2021.