

IWONA BIL-LULA<sup>1, B–D</sup>, PATRYK MATUSZEK<sup>2, B, C</sup>, THOMAS PFEIFFER<sup>3, B</sup>,  
MIECZYSLAW WOŹNIAK<sup>1, A, E, F</sup>

## Lyme Borreliosis – the Utility of Improved Real-Time PCR Assay in the Detection of *Borrelia burgdorferi* Infections

<sup>1</sup> Department of Clinical Chemistry, Wrocław Medical University, Poland

<sup>2</sup> Euroimmun, Wrocław, Poland

<sup>3</sup> Euroimmun AG, Lubeck, Germany

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

### Abstract

**Background.** Infections of *Borrelia burgdorferi sensu lato* reveal clinical manifestations affecting numerous organs and tissues. The standard diagnostic procedure of these infections is quite simple if a positive history of tick exposure or typical erythema migrans appears. Lack of unequivocal clinical symptoms creates the necessity for further evaluation with laboratory tests.

**Objectives.** This study discusses the utility of a novel, improved, well-optimized, sensitive and highly specific quantitative real-time PCR assay for the diagnostics of infections caused by *Borrelia burgdorferi sensu lato*.

**Material and Methods.** We designed an improved, specific, highly sensitive real-time quantitative polymerase chain reaction (RQ-PCR) assay for the detection and quantification of all *Borrelia burgdorferi* genotypes. A wide validation effort was undertaken to ensure confidence in the highly sensitive and specific detection of *B. burgdorferi*.

**Results.** Due to high sensitivity and great specificity, as low as  $1.6 \times 10^2$  copies of *Borrelia* per mL of whole blood could be detected. As much as 12 (3%) negative ELISA IgM results, 14 (2.8%) negative results of Line blot IgM, 11 (3.1%) and 7 (2.7%) of negative ELISA IgG and Line blot IgG results, respectively, were positive in real-time PCR.

**Conclusions.** The data in this study confirms the high positive predictive value of real-time PCR test in the detection of *Borrelia* infections (Adv Clin Exp Med 2015, 24, 4, 663–670).

**Key words:** borreliosis, RQ-PCR, whole blood.

Lyme disease (LD), recognized as a multisystem infection, was primarily described in the late 20<sup>th</sup> century [1]. It is caused by the bite of a *Ixodes ricinus* complex tick infected with *Borrelia burgdorferi sensu lato* [2]. Infections of *Borrelia burgdorferi sensu lato* reveal clinical manifestations affecting numerous organs and tissues, dependent on the different species of *Borrelia* [3–6]. The group of spirochetes, usually called *Borrelia burgdorferi sensu lato*, includes 11 different genospecies, from which 3 are frequently observed in Europe: *B. burgdorferi sensu stricto*, *Borrelia afzelii* and *Borrelia garinii* [7, 8]. All 3 genospecies of *Borrelia* are pathogenic in Europe and can be a potential infection agent for specific high risk

subjects such as foresters [6, 8, 9]. The most common clinical manifestation (80% of infected subjects) of early infection of *Borrelia burgdorferi sensu lato* is localized erythema migrans, which may be followed by disseminated infection affecting the skin, nervous system, heart or joints before late infection [1, 4, 10, 11]. Nevertheless, LD may also be latent, without unequivocal clinical symptoms or may present unspecific symptoms such as headache, myalgia, arthralgias or fever [12, 13]. The standard diagnostic procedure of these infections is quite simple if a positive history of tick exposure or typical erythema migrans (clinical hallmark) appears. Further laboratory testing in these cases is not required. Genetic diversity and

differential expression of *B. burgdorferi sensu lato* genes in subjects have important implications for the development of molecular assays and serologic tests in the laboratory diagnosis of LB. Serological testing is based mainly on the detection of antibodies class IgM and IgG using ELISA, EIA and line blot tests [14–17]. Nevertheless, serologic tests are often unsuitable or insufficient as they present high cross-reactivity with antigens of other bacteria, viruses and mammalian tissues; they cannot distinguish between primary or recurrent infections and are not useful during the “window period”. Moreover, serological tests can be only use in subjects not vaccinated against *Borrelia* spp. [11]. Therefore, attempts are being made to introduce some new diagnostic tests for the detection and monitoring of infections with *Borrelia burgdorferi sensu lato* around the world. There are some reports confirming the utility of PCR assays in the detection of *Borrelia* spp. in cerebrospinal fluid (CSF), synovial fluid, skin biopsies and urine samples [18–20]. Taking into account that the etiologic agent of LD, *Borrelia burgdorferi sensu lato*, was recovered first from the tick *Ixodes dammini* in the year 1982 [2] and then from a skin biopsy, CSF and blood specimens of patients with LB around the world [2, 21–25], the main aim of this study was to discuss the utility of introduction of a well-optimized, sensitive and highly specific quantitative real-time PCR assay into the diagnostics of *Borrelia burgdorferi sensu lato* infections.

## Material and Methods

### Clinical Samples

Five hundred seventy seven subjects participated in this study. All of the subjects belonged to the group at high risk of developing Lyme disease due to infection of *Borrelia burgdorferi sensu lato*. Five hundred sixty seven of them were wood workers and 10 subjects could be potentially affected by the infection on the basis of medical history. A written informed consent was obtained from the subjects for the collection of blood samples. The study was approved by the local Ethics Committee of Wroclaw Medical University (KB: 371/2008). The study subjects were informed about the purpose and principles of this study. All subjects were screened by ELISA, Western blot (the confirmation test) and real-time PCR tests for the detection of exposure/presence of the pathogen.

Whole blood (EDTA and for serum separation) was collected from the subjects. Samples of the whole blood for isolation of DNA were frozen

immediately after collection in  $-20^{\circ}\text{C}$ . Samples of the whole blood for serological testing were centrifuged 10 min  $2500 \times g$  and tested immediately after collection or frozen in aliquots in  $-20^{\circ}\text{C}$ .

### Screening Examination of Serum Samples

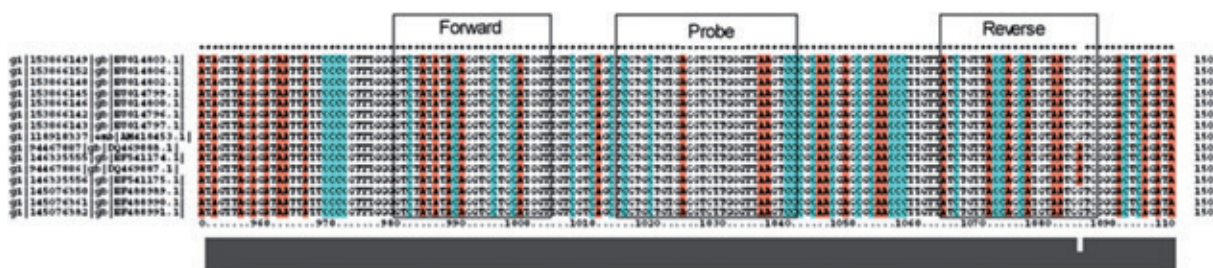
Serum samples collected from all subjects were screened for the presence of antibodies against *Borrelia* spp. in class IgM and IgG. The antibodies were detected using Anti-Borrelia plus VIsE ELISA (IgG) and Anti-Borrelia ELISA (IgM) (Euroimmun, Wrocław, Poland). Positive results were verified using Line blot tests (Euroline Borrelia-RN-AT test, Euroimmun), according to the manufacturer's instructions. The IgG line blot used included the native antigens p83, OspC (Ba, Bb, Bg) antigens, p39, flagellin and recombinant VIsE (Bb) antigens from different species of *Borrelia* (*B. afzelii*, *burgdorferi*, *garninii*), 2 extracted lipid fractions and selected recombinant antigens p58, p21, p20, p19 and p18, while the corresponding ELISA tests used detergent extracts of all 3 species with additional VIsE of *B. burgdorferi*. Evaluation of the test strips was performed using the fully automated EUROLineScan software (Euroimmun).

### Isolation of DNA from Clinical Samples

DNA was extracted from the whole blood using spin columns from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. From a starting amount of 200  $\mu\text{L}$  of whole blood, 200  $\mu\text{L}$  of extracted DNA was collected. The extracted DNA was frozen at  $-20^{\circ}\text{C}$  until testing. The DNA obtained from the whole blood samples was analyzed for detection of *Borrelia burgdorferi sensu lato* by means of real-time PCR assay.

### Real-Time PCR for Detection of *Borrelia* spp.

The DNA extracted from the whole blood samples was screened for the presence of *Borrelia* spp.-DNA. The time from the tick bite to the real-time PCR performance had a wide range, from several days to several months. The primers and probe for the real-time PCR detection system were placed within the region of the gene coding ribosomal RNA (*16SrRNA*) of *Borrelia* spp. The most conservative and most specific region for *Borrelia* spp. was assessed by means of a comparison of *16SrRNA* sequences obtained from 15 different gene-species of *Borrelia* spp. using ClustalX 2.0.6



**Fig. 1.** Bioinformatic analysis of the conservative region in the *16SrRNA* gene sequence using ClustalX 2.0.6 tool. The frames indicate the sequences of primers and probe

(Fig. 1). The DNA sequences of 16sRNAs were obtained from the National Center for Biotechnology Information (NCBI) database. The primer and probe design was based on the sequences of the following genotypes: Tom3401, Mng3602, isolate Ir-2200, Nov14506, Nov7006, Nov9906, Nov11506, Nov1105, isolate BUL-1, isolate BUL-4, isolate BUL-6, isolate BUL-10, isolate SCCH-9, isolate SCCH-13, isolate SI-15 (accession numbers: DQ469887.1, DQ469888.1, AM418453.1, EF541174.1 EF541175.1, EF488989.1, EF488990.1, EU014796.1, EU014797.1, EU014799.1, EU014800.1, EU014802.1, EU014803.1, EF488991.1, EU014806.1). The following sequences of primers and probe were selected: Forward: GGC AAC CCT AAG GTG AAG GC, Reverse: GGT GAG CCA GGC CAT CAC TA, Probe: -FAM-CAT GGC AAG AAA GTG CTC GGT GCC T-BBQ. A gene of B-globulin served as an internal control. The sequences of the primers and probe were derived from the previous report [28]. Yak and BBQ were used for labeling the probe. Prior to the experimental testing, the primer and probe sequences were tested by a standard nucleotide-nucleotide BLAST tool for the absence of homology with any other relevant organism. None of the primers and probes presented significant homology to DNA other than intended target.

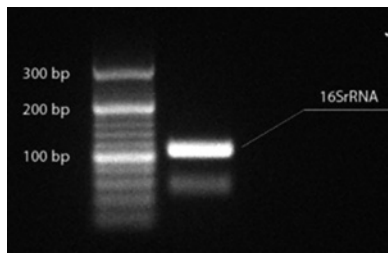
Quantitative amplification was performed on a Real Time 7000 PCR System v.1.1 (Life Technologies, Foster City, USA) in a 96-well format by using TaqMan-based chemistry. In brief, the optimized master mix consisted of 25  $\mu$ L of  $\mu$ L 2  $\times$  PCR Master Mix for Probe Assays (Eurogentec, Seraing, Belgium), 2  $\mu$ L of forward primer *16SrRNA* (800 nM final conc.), 2  $\mu$ L of reverse primer *16SrRNA* (400 nM final conc.), 1  $\mu$ L of probe *16SrRNA* (200 nM final conc.), 2  $\mu$ L of forward and reverse primers for B-globulin (27 nM final conc.; internal control), 1  $\mu$ L of probe for B-globulin (200 nM final conc.) and 5  $\mu$ L of target DNA in a final total volume of 50  $\mu$ L. Real-time PCR was performed under the following conditions: 1 cycle at 50°C for 2 min for AmpErase UNG activation, then 95°C for 10 min, 45 cycles of 95°C for 15 s, and 60°C

for 1 min. To reduce the risk of false-positive results due to contamination with PCR products, dTTP was partially replaced by dUTP in the reaction master mixture and a dUTP glycosylase step was performed prior to each PCR. Each DNA sample was analyzed in duplicate. Negative controls (no template, no amplification) were included in each assay. DNA isolates derived from *Borrelia afzelii* (strain VS461/ET139, reference no. DSM10508, derived from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures) served as a positive control.

## Preparation of Control DNA and Generation of Calibration Curve

To quantify *Borrelia* spp. load in clinical samples, an external standard curve was generated. Preparation of the exogenous standard curve was based on 10-fold serial dilutions of spectrophotometrically quantified standards (amplicons 108 bp cloned and amplified in a pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector from a TOPO TA Cloning<sup>®</sup> kit (Invitrogen, Life Technologies, Foster City, USA), according to the manufacturer's instructions. In brief, the DNA of *Borrelia afzelii* (strain VS461/ET139) was amplified by PCR. PCR was performed as a singleplex assay in a total volume of 25  $\mu$ L, containing 5  $\mu$ L of Green GoTaq Flexi Buffer – 5x (Promega, Madison, USA), 3 mM of 25 mM MgCl<sub>2</sub>, 2 mM dNTP-Mix, 2.5  $\mu$ L 10  $\mu$ M forward: GTCTATATACAGGTGCTGCATGGT and reverse: CACCATTACATGCTGGTAACAGAT primers, 0.4  $\mu$ L GoTaq DNA Polymerase (5 U/ $\mu$ L), 10.1  $\mu$ L distilled water and 1  $\mu$ L target nucleic acid. The amplification was performed according to the following protocol: 94°C for 2 min; 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; followed by 72°C for 10 min. PCR products stained with ethidium bromide were visualized after electrophoresis in 2% agarose gel (Fig. 2).

Plasmid DNA was purified with a Plasmid Midi Kit (Qiagen), according to the manufacturer's instructions. The proper insertion of the PCR product into the plasmid was checked by the



**Fig. 2.** Product of amplification of *Borrelia afzelii*-DNA (stain VS461/ET139). Line 1 – molecular marker; line 2 – product 108 bp

sequencing of the plasmids (obtained from 3 randomly selected colonies of transfected *E. coli* XL-2 (Agilent Technologies Division, Santa Clara, USA) with primers surrounding the insert: M13 forward: GTAAAACGACGGCCAG and M13 reverse: CAGGAAACAGCTATGAC. The sequence obtained was compared to a *16SrRNA* control sequence from *Borrelia afzelii* strain VS461/ET139 by ClustalX 2.0.6. software. All 3 sequences were similar to the control sequence (Fig. 3). Subsequently, the plasmid concentration and purity were determined using a NanoPhotometer (Implen GmbH). The viral copy number was calculated based on the known molecular weight of the plasmids and amplicons. Ten-fold serial dilutions of the plasmid ranging from  $10^6$  to  $10^{1/5}$   $\mu$ L were amplified in triplicate for the construction of standard curves ( $R^2 = 0.9988$ ). The sensitivity of the real-time PCR assay permitted reliable detection of copies  $2 \times 10^2$  copies/mL of the medium investigated.

## Results

### Antibodies Against *Borrelia* spp.

Five hundred seventy seven subjects (491 male and 86 female) were included in the study. The median age of the participants was 45 (20–65) years. According to the recommendations of the Centers for Disease Control and Prevention (CDC, Atlanta, USA, 2005) for the diagnosis of *Borrelia* spp. infections, the diagnosis of the infection was based on clinical symptoms (erythema migrans, palsy of facial nerve or arthritis), the patient’s medical history and an assessment of the risk of exposure to infected ticks combined with diagnostic tests

including the assessment of antibodies to *Borrelia* spp. class IgM and IgG.

ELISA tests for the detection of IgG antibodies to *Borrelia* spp. revealed 202 (33%) positive subjects, 350 (60.7%) negative and 25 (4.3%) results were considered as borderline results. During IgM testing, 122 (21.1%) subjects were positive, while 411 (71.2%) and 44 (7.6%) were negative or borderline results, respectively. Line blot tests confirmed 221 (38.3%) positive results, 257 (44.5%) were negative and 99 (17.6%) constitute the borderline results in class IgG, as described previously.

### Incidence of *Borrelia* spp. Infection According to Real-Time PCR Detection

Among the 577 subjects from the group at high risk of *Borrelia* spp. infection, only 18 (3.1%) subjects were positive for *16SrRNA*-DNA in the whole blood. Bacterial load in clinical samples was as low as  $2.0 \times 10^2$ – $4.6 \times 10^3$  copies/mL. Eight of the 18 (44.4%) positive subjects were also positive for IgG antibodies in one or both ELISA/line blot tests and 3 of them (16.6%) were positive in real-time PCR and doubtful in the blot. As much as 7 of 18 positive subjects (in real-time PCR test) were negative for IgG antibodies in both serological tests. In parallel, 6 of the 18 (33.3%) subjects found positive by the real-time PCR method were also positive by ELISA/line blot IgM tests, but as much as 12 of the 18 (66.6%) were positive for *Borrelia* spp. DNA and negative in the serologic tests (Table 1).

As little as 4% of the positive ELISA results and 3.2% of the positive line blot tests were positive in the real-time PCR assay. Nevertheless, the correlation of all diagnostic tests used in this study showed that as much as 12 (3%) of the negative ELISA IgM results, 14 (2.8%) negative results of the line blot IgM, 11 (3.1%) and 7 (2.7%) of the negative ELISA IgG and line blot IgG results, respectively, were positive in real-time PCR (Table 2).

## Discussion

Diagnosis of *Borrelia* spp. infections generates a lot of problems. According to the recommendations of the Centers for Disease Control



**Fig. 3.** The comparison of the insert sequence obtained from 3 different colonies of *E. coli* XL-2 transfected with plasmid containing *Borelia afzelii* *16srRNA* PCR-product (9<sub>-</sub>–; 9<sub>-</sub>–; 10<sub>+</sub>–) with a *16SrRNA* control gene of *Borrelia afzelii* (VS461 ET139)

**Table 1.** Comparison of real-time PCR positive samples with results of serologic tests

Subject no.	Diagnostic method					
	real-time PCR		serologic tests			
	copies no./mL	IC	IgG ELISA	IgG Blot	IgM ELISA	IgM Blot
1	1164	+	-	-	-	-
2	222	+	+	+	+	(+)
3	484	+	-	(+)	-	-
4	228	+	-	+	-	-
5	316	+	-	-	-	-
6	474	+	+	+	+	(+)
7	340	+	+	+	+	+
8	360	+	-	-	-	-
9	1318	+	-	(+)	-	-
10	4658	+	+	+	-	-
11	2234	+	+	+	+	(+)
12	1026	+	+	+	-	-
13	1322	+	-	(+)	-	-
14	168	+	-	-	-	-
15	1612	+	-	-	+	-
16	1842	+	+	+	+	-
17	4566	+	-	-	-	-
18	296	+	-	-	-	-

IC – internal control (DNA of B-globulin)

and Prevention, the diagnostics of *Borrelia* spp. infections should be based mainly on clinical symptoms, such as: erythema migrans, palsy of the facial nerve or arthritis, the patient's medical history and an assessment of the risk of exposure to infected ticks combined with diagnostic tests including the assessment of antibodies to *Borrelia* spp. class IgM and IgG. The common diagnostic tests for *Borrelia* spp. are divided into 2 different groups. The first one, called direct tests, are able to detect the pathogen in the clinical material (culture collection, microscopic methods, PCR and real-time PCR) and the second group includes indirect tests, which make it possible to assess the contact of the subject with the pathogen (serum antibodies). The main aim of this study was to develop a sensitive, specific, and rapid TaqMan PCR assay for the detection of *B. burgdorferi sensu lato* in whole blood samples collected from subjects at a high risk of infection with *Borrelia* spp. and an assessment of the utilization of this assay to complement the immunoserologic methods to diagnose LD.

The main goal of the serological tests is the detection of antibodies to *Borrelia* spp. in serum. Confirmation of anti-*Borrelia* antibodies in serum carries an important confirmation that the subject was exposed to infected ticks in the distant or recent past. The diagnostic value of serological tests is limited due to "the window period", in which no antibodies can be detected with any diagnostic tests. In addition, a positive result of a serologic test (even IgM) does not always correlate with an active infection. Furthermore, the titer of antibodies usually does not correlate with the progress of the infection. The presence of the antibodies in serum can be detected many years after primary infection; hence the diagnostic value of these testes in the early detection of infection is low. The diagnostic problem of ELISA tests is also the large cross-reactivity to antigens commonly observed in other bacteria, viruses and mammalian tissues such as p41, p58-60, p66, p68, p71, p73, flagellin etc. [26, 27]. The line blot tests confirmed that more than 8% of the positive results of the ELISA in class IgG and

**Table 2.** Comparison of results obtained by means of ELISA/line blot IgM (A), IgG (B) and real-time PCR tests in 577 clinical samples

A.									
Diagnostic method									
PCR	Elisa IgM				PCR	line blot IgM			
	positive	negative	pos/neg	total		positive	negative	pos/neg	total
Positive	6 (4.9%)	12 (2.9%)	0 (%)	18	positive	1 (1.6%)	14 (2.8%)	3 (9.6%)	18
Negative	116 (95.1%)	399 (97.0%)	44 (100%)	559	negative	59 (98.3%)	472 (97.1%)	28 (90.3%)	556
Pos/Neg	0 (0%)	0 (0%)	0 (%)	0	pos/neg	0 (0%)	0 (0%)	0 (%)	0
Total	122	411	44	577	total	60	486	31	577
B.									
Diagnostic method									
PCR	Elisa IgG				PCR	line blot IgG			
	positive	negative	pos/neg	total		positive	negative	pos/neg	total
Positive	7 (3.5%)	11 (3.1%)	0 (%)	18	positive	8 (3.6%)	7 (2.7%)	3 (3%)	18
Negative	195 (96.5%)	339 (96.8%)	25 (100%)	559	negative	213 (96.4%)	250 (97.3%)	96 (96.9%)	559
Pos/Neg	0 (0%)	0 (0%)	0 (%)	0	pos/neg	0 (0%)	0 (0%)	0 (%)	0
Total	202	350	25	577	total	221	257	99	577

36% positive results of the ELISA in class IgM were false positive. This means that the line blot test should be always introduced to reduce the number of false positive results and to prevent the introduction of unnecessary, 3–4 week long drug therapy. *B. burgdorferi sensu lato* expresses different surface proteins in adaptation to various microenvironments. The spirochete expresses OspA but not OspC when residing in the midguts of unfed ticks. Notwithstanding, during a blood meal by the tick, some spirochetes stop expressing OspA and instead express OspC [28–31]. Some genes of *B. burgdorferi sensu lato* are expressed only in a mammalian host or have significantly upregulated expression in that environment, for example VlsE, DbpA, BBK32, Erp, and Mlp proteins [30, 32–36]. Therefore, many false positive results may appear during in ELISA/line blot test and detection of bacteria genomic DNA may play an important role in the diagnosis of *Borrelia* infections. The diagnostic problem of the serologic tests is also the large amount of false negative results. This can be due to “the window period” in which IgM antibodies are not produced yet or due to the forming of antigen-antibodies complexes which cannot be detected by means of ELISA/line blot tests. Therefore, the real-time PCR assay would be valuable in testing patients early in the disease, before an antibody response develops, and in cases where the patient does not have classic symptoms, a diagnosis

based on the real-time PCR method seems to be useful. In addition, the TaqMan real-time PCR assay would be useful in guiding therapy in patients who have had prior exposure to the organism, where the interpretation of immunoserologic results would be difficult for some reason. The increasing use of RQ-PCR in clinical diagnostics in recent years has made this technique an essential tool in laboratory detection of many infections. This technique presents many advantages, including improved speed, high sensitivity and specificity and low intra- and inter-assay variability [37]. The use of probes allows for precise measurement and monitoring of pathogen DNA replication in clinical samples [21]. In this study, the utility of the real-time PCR assay developed in our laboratory in the detection of *Borrelia* spp. DNA was confirmed. An average of 3% false negative ELISA/line blot results were detected using real-time PCR. Due to high sensitivity and great specificity, as low as  $1.6 \times 10^2$  copies of *Borrelia* per mL of whole blood could be detected. The low sensitivity of the TaqMan assay in comparison to serological tests could be a reflection of the lack of spirochetemia or transient spirochetemia or a low level of spirochetes in the blood. In addition, both negative and positive controls and an internal control were included in each run to determine whether or not inhibitory substances are present in the patient’s clinical sample or whether false positive results could appear during

amplification. Therefore, we excluded the possibility of low test sensitivity due to the presence of PCR inhibitors in the host blood. The data in this study confirms the high positive predictive value of real-time PCR tests in the detection of *Borrelia* infections. Nevertheless, the low detectability of *Borrelia* spp. in whole blood samples showed that whole blood is not a main area of infection and the choice of other tissues or body fluids would be more appropriate [20]. Due to the great analytical sensitivity and specificity of the real-time PCR assay presented in this study, we speculate that this test could be much more useful for the detection of *Borrelia burgdorferi sensu lato* infections localized in tissues such as skin or other body fluids such as cerebrospinal fluid or synovial fluid. In our opinion, real-time PCR assay should not be the first line test in the diagnosis of *Borrelia* infections. Nevertheless,

real-time PCR assay as a highly sensitive and specific test could be used in the detection of early infection of *Borrelia* spp. in localized tissue and can be used to complement immunoserologic methods to diagnose infections of *Borrelia burgdorferi sensu lato*.

In conclusion, due to the many disadvantages of the ELISA test, line blot confirmation tests are necessary to reduce the number of ELISA false positive results. In addition, the real-time PCR assay would be valuable in testing patients in the early period after exposure, before an antibody response develops, if the patient does not have any clinical symptoms of infection and in early disseminated diseases such as neuroborreliosis and carditis. Furthermore, the assay presented in this study would be useful in patients exposed to *Borrelia burgdorferi sensu lato*, in whom the interpretation of immunoserologic results is difficult.

## References

- [1] Steere AC: Lyme disease. *N Engl J Med* 2001, 345, 115–125.
- [2] Žáková A, Nejezchlebová H, Bartoňková N, Rašová T, Kučerová H, Norek A, Ovesná P: Activity of the tick *Ixodes ricinus* monitored in a suburban park in Brno, Czech Republic, in association with the evaluation of selected repellents. *J Vector Ecol* 2013, 38, 295–300.
- [3] Stinco G, Ruscio M, Bergamo S, Trotter D, Patrone P: Clinical features of 705 *Borrelia burgdorferi* seropositive patients in an endemic area of northern Italy. *Sci World J* 2014, 4, 414–505.
- [4] Mosshammer D, Hlobil H, Joos S, Reichert W: A patient with Lyme arthritis presenting in general practice. *Practitioner* 2013, 257, 25–27.
- [5] Esposito S, Bosis S, Sabatini C, Tagliaferri L, Principi N: *Borrelia burgdorferi* infection and Lyme disease in children. *Int J Infect Dis* 2013, 17, 153–158.
- [6] Grygorczuk S, Péter O, Kondrusik M, Moniuszko A, Zajkowska J, Dunaj J, Żukiewicz-Sobczak W, Pancewicz S: Assessment of the frequency of different *Borrelia burgdorferi sensu lato* species in patients with Lyme borreliosis from north-east Poland by studying preferential serologic response and DNA isolates. *Ann Agric Environ Med* 2013, 20, 21–29.
- [7] Rauter C, Hartung T: Prevalence of *Borrelia burgdorferi sensu lato* genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. *Appl Environ Microbiol* 2005, 71, 7203–7216.
- [8] Hanincova K, Mukherjee P, Ogden NH, Margos G, Wormser GP, Reed KD, Meece JK, Vandermause MF, Schwartz I: Multilocus sequence typing of *Borrelia burgdorferi* suggests existence of lineages with differential pathogenic properties in humans. *PLoS One* 2013, 8, e73066.
- [9] Agüero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP: Diagnosis of Lyme borreliosis. *Clin Microbiol Rev* 2005, 18, 484–509.
- [10] Stanek G, Strle F: Lyme borreliosis. *Lancet* 2003, 362, 1639–1647.
- [11] Fawcett PT, Rose CD, Maduskuie V: Long-term effects of immunization with recombinant lipoprotein outer surface protein a on serologic test for Lyme disease. *Clin Diagn Lab Immunol* 2004, 11, 808–810.
- [12] Smith RP, Schoen RT, Rahn DW, Sikand VK, Nowakowski J, Parenti DL, Holman MS, Persing DH, Steere AC: Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. *Ann Intern Med* 2002, 136, 421–428.
- [13] Wormser GP, Nadelman RB, Nowakowski J, Schwartz I: Asymptomatic *Borrelia burgdorferi* infection. *Med Hypotheses* 2001, 57, 435–438.
- [14] Steere AC, McHugh G, Damle N, Sikand VK: Prospective study of serologic tests for Lyme disease. *Clin Infect Dis* 2008, 47, 188–195.
- [15] Liu ZY, Hao Q, Hou XX, Jiang Y, Geng Z, Wu YM, Wan KL: A study of the technique of Western blot for diagnosis of Lyme disease caused by *Borrelia afzelii* in China. *Biomed Environ Sci* 2013, 26, 190–200.
- [16] Hinterseher I, Gäbel G, Corvinus F, Lück C, Saeger HD, Bergert H, Tromp G, Kuivaniemi H: Presence of *Borrelia burgdorferi sensu lato* antibodies in the serum of patients with abdominal aortic aneurysms. *Eur J Clin Microbiol Infect Dis* 2012, 31, 781–789.
- [17] Robertson J, Guy E, Andrews N, Wilske B, Anda P, Granstrom M, Hauser U, Moosmann Y, Sambri V, Schellekens J, Stanek G, Gray J: A European multicenter study of immunoblotting in serodiagnosis of Lyme borreliosis. *J Clin Microbiol* 2000, 38, 2097–2102.
- [18] Bergmann AR, Schmidt BL, Derler AM, Aberer E: Importance of sample preparation for molecular diagnosis of Lyme borreliosis from urine. *J Clin Microbiol* 2002, 40, 4581–4584.

- [19] **Liveris D, Wang G, Girao G, Byrne DW, Nowakowski J, McKenna D, Nadelman R, Wormser GP, Schwartz I:** Quantitative detection of *Borrelia burgdorferi* in 2-millimeter skin samples of erythema migrans lesions: correlation of results with clinical and laboratory findings. *J Clin Microbiol* 2002, 40, 1249–1253.
- [20] **Lynn I, Reed KD, Mitchell PD:** A LightCycler TaqMan assay for detection of *Borrelia burgdorferi sensu lato* in clinical samples. *Diagn Microb Infect Dis* 2007, 57, 137–143.
- [21] **Mackay IM, Arden KE, Nitsche A:** Real-time PCR in virology. *Nucleic Acids Res* 2002, 30, 1292–1305.
- [22] **Radzišauskienė D, Ambrozaitis A, Marciuškienė E:** Delayed diagnosis of Lyme neuroborreliosis presenting with abducens neuropathy without intrathecal synthesis of *Borrelia* antibodies. *Medicina (Kaunas)* 2013, 49, 89–94.
- [23] **Schwan TG, Piesman J:** Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. *J Clin Microbiol* 2000, 38, 382–388.
- [24] **Fomenko NV, Livanova NN, Romanova EV, Karavaeva Iulu, Panov VV, Chernousova NIa:** Detection of DNA of borrelia circulating in Novosibirsk region. *Zh Mikrobiol Epidemiol Immunobiol* 2006, 7, 22–28.
- [25] **Magnarelli LA, Williams SC, Norris SJ, Fikrig E:** Serum antibodies to *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti* in recaptured white-footed mice. *J Wildl Dis* 2013, 49, 294–302.
- [26] **Rawlins ML, Gerstner C, Hill HR, Litwin CM:** Evaluation of a Western blot method for the detection of *Yersinia* antibodies: evidence of serological cross-reactivity between *Yersinia* outer membrane proteins and *Borrelia burgdorferi*. *Clin Diagn Lab Immunol* 2005, 12, 1269–1274.
- [27] **Magnarelli LA, Fikrig E, Padula SJ, Anderson JF, Flavell RA:** Use of recombinant antigens of *Borrelia burgdorferi* in serologic tests for diagnosis of Lyme borreliosis. *J Clin Microbiol* 1996, 34, 237–240.
- [28] **Fikrig E, Feng W, Aversa J, Schoen RT, Flavell RA:** Differential expression of *Borrelia burgdorferi* genes during erythema migrans and Lyme arthritis. *J Infect Dis* 1998, 178, 1198–1201.
- [29] **de Silva AM, Fikrig E:** *Borrelia burgdorferi* genes selectively expressed in ticks and mammals. *Parasitol Today* 1997, 13, 267–270.
- [30] **Ohnishi J, Piesman J, de Silva AM:** Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. *Proc Natl Acad Sci USA* 2001, 98, 670–675.
- [31] **Schwan TG, Piesman J:** Vector interactions and molecular adaptations of Lyme disease and relapsing fever spirochetes associated with transmission by ticks. *Emerg Infect Dis* 2002, 8, 115–121.
- [32] **Crother TR, Champion CI, Wu XY, Blanco DR, Miller JN, Lovett MA:** Antigenic composition of *Borrelia burgdorferi* during infection of SCID mice. *Infect Immun* 2003, 71, 3419–3428.
- [33] **Fikrig E, Feng W, Barthold SW, Telford III SR, Flavell RA:** Arthropod- and host-specific *Borrelia burgdorferi* bbk32 expression and the inhibition of spirochete transmission. *J Immunol* 2000, 164, 5344–5351.
- [34] **Hubner A, Yang X, Nolen DM, Popova TG, Cabello FC, Norgard MV:** Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. *Proc Natl Acad Sci USA* 2001, 98, 12724–12729.
- [35] **Miller JC, von Lackum K, Babb K, McAlister JD, Stevenson B:** Temporal analysis of *Borrelia burgdorferi* Erp protein expression throughout the mammal-tick infectious cycle. *Infect Immun* 2003, 71, 6943–6952.
- [36] **Yang X, Popova TG, Hagman KE, Wikel SK, Schoeler GB, Caimano MJ, Radolf JD, Norgard MV:** Identification, characterization, and expression of three new members of the *Borrelia burgdorferi* Mlp (2.9) lipoprotein gene family. *Infect Immun* 1999, 67, 6008–6018.
- [37] **Bil-Lula I, De Franceschi N, Pawlik K, Woźniak M:** Improved real-time PCR assay for detection and quantification of all 54 known types of human adenoviruses in clinical samples. *Med Sci Monit* 2012, 18, 221–228.

### Address for correspondence:

Iwona Bil-Lula  
Department of Clinical Chemistry  
Wrocław Medical University  
Borowska 211  
50-556 Wrocław  
Poland  
Tel.: +48 71 78 40 621  
E-mail: iwona.bil-lula@umed.wroc.pl

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