Development and evaluation of a two-step multiplex TaqMan real-time PCR assay for detection/quantification of different genospecies of *Borrelia burgdorferi* sensu lato

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Abstract

Nowadays, at least four clinically important *B. burgdorferi* sensu lato (s.l.) genospecies (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto (s.s.) and *B. lusitaniae*) circulate in Portugal. Each genospecies have different tropism that result in a diverse array of clinical manifestations. The standard diagnostic procedure used is normally simple, nevertheless, during the “window-period” phase, in which specific antibodies cannot yet be detected, diagnosis becomes difficult, and calls for reliable, sensitive and specific laboratory methods, such as molecular tests. The aim of this study was to develop and evaluate a multiplex *TaqMan* real-time PCR assay to infer the presence of *B. burgdorferi* s.l. genospecies in clinical and vector-derived samples. The assay consists of two steps: (i) a first duplex real-time PCR targeting both *flaB* of *B. burgdorferi* s.l., and an internal control (18S rDNA for tick samples or the mammal *β-actin* gene for clinical samples); and (ii) a second tetraplex real-time PCR targeting the *flaB* gene of *B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. lusitaniae*.

The first step revealed a high specificity and sensitivity, allowing the detection of as low as 20 genome equivalents (GE) of *B. burgdorferi* s.l. from isolated cultures, clinical samples and ticks. The second step revealed high specificity, but a slightly lower sensitivity (2×10^2 GE) for detection of *B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. lusitaniae* in purified DNA extracts, and more particularly when testing cerebrospinal fluid (CSF) samples. Nonetheless, both real-time PCR protocols were developed to be applied at the beginning of the infection, to improve early diagnosis of Lyme borreliosis (LB), where detection of *Borrelia* should not rely on the use of CSF samples. The assay here described is of special interest for the analysis of both environmental and clinical samples, being advantageous in the former phase screening of Lyme borreliosis, when the efficiency of serologically based diagnoses may be seriously compromised.
Keywords: Lyme borreliosis, molecular diagnosis, multiplex TaqMan real-time PCR, *Borrelia burgdorferi* s.l. species differentiation

Introduction

Lyme borreliosis (LB) is known as the most common vector-borne disease in both Europe and North America (ECDC, 2016). The number of cases in the last two decades points to 360 000 cases in Europe (with a marked increase) (ECDC, 2014), and approximately 300 000 cases in the USA, between 1995 and 2015 (Lindgren & Jaenson, 2006; Hinckley et al., 2014). Nearly all human cases in Europe are caused by three members of the *B. burgdorferi* sensu lato (s.l.) complex, namely *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto (s.s.) (Rizzoli et al., 2011), all of which are transmitted through the bite of *Ixodes ricinus* ticks. In the USA, *B. burgdorferi* s.s. was the only species associated to LB. However, more recently *Candidatus B. mayonii* was also identified as the causative agent of LB (Stanek et al., 2012, Pritt et al., 2016).

The clinical manifestations of LB are wide-ranging, and linked with differential bacterial tropisms for distinct tissues or systems (van Dam et al., 1993; Balmelli & Piffaretti, 1995). Early localized infections typically result in a rash known as erythema migrans (EM), from which spirochetes can disseminate to the central and peripheral nervous systems, joints, and other organs. Infections with *B. burgdorferi* s.s. are usually associated with arthritis, while those caused by *B. garinii* and *B. afzelii* are usually accompanied by neurological and skin complications (e.g. Bell’s palsy, encephalopathy and acrodermatitis chronica atrophicans – ACA), respectively (van Dam et al., 1993). Nevertheless, LB may also remain latent, without an unequivocal clinical presentation, or translate into a clinical presentation including unspecific symptoms such as headache, myalgia, arthralgia or fever (Smith et al., 2002; Steere et al., 2003).
Currently, in case the affected individual reports recent tick bites, or shows symptoms typical of EM the standard diagnosis is clinical. However, when a laboratory confirmation of a clinical diagnosis is required, several direct as well as indirect methods may be used. Direct detection of *B. burgdorferi* s.l. spirochetes may be carried out by examination of specimens under dark-field microscope, detection of bacterial DNA using conventional polymerase chain reaction (PCR), and culture in individual cases where the clinical picture suggests LB despite a negative antibody assay (e.g. in atypical EM or in suspected acute neuroborreliosis without detection of intrathecal antibodies) (Gaumond et al., 2006, Marques, 2015). However, this is a very time-consuming method characterized by a low sensitivity, especially in body fluids (Wilske, 2007). Indirect diagnostic methods, involving the detection of IgM/IgG anti-*B. burgdorferi* s.l. antibodies, may be performed by ELISA, EIA and immunoblot tests (Steere et al., 2008; Hinterseher et al., 2012; Liu et al., 2013). Despite their generalized use, the available serological tests are frequently unsuitable or insufficient for a conclusive diagnosis due to high levels of cross-reactivity between anti-*B. burgdorferi* s.l. antibodies and non-*Borrelia* antigens, which compromise the distinction between primary and recurrent infections, and the identification of *bona-fide* infections during the immunological window period (Marques, 2015).

PCR-based assays have been proven useful to screen for *B. burgdorferi* s.l. cases in an early phase, before the development of an immune response and production of IgM/IgG antibodies. These assays allow the detection of the spirochete’s genome in biological samples without requiring their cultivation by targeting chromosomal genes such as *recA*, *flaB*, plasmid genes *ospA*, *ospC*, 16S rDNA, or the *rrs-rrlA* intergenic spacer (16S-23S IGS) (Schmidt, 1997; Lebech, 2002).
The aim of this study was to develop an easy-to-use TaqMan real-time PCR assay for the
detection of *B. burgdorferi* s.l. spirochetes, also allowing the differentiation of clinically-
relevant species of the complex.

**Material and methods**

**TaqMan probes and flanking primers**

A multiple sequence alignment of *flaB* [located in the bacterial linear chromosome, which encodes a 41-kDa flagellin protein (Wang, 1999)] reference sequences retrieved from GenBank was created using Mafft 7 (Katoh & Standley, 2013). The flagellin-coding sequences used included those of *B. burgdorferi* s.s. B31 (accession number CP009656.1), *B. garinii* SZ (accession number CP007564.1), *B. afzelii* HLJ01 (accession number CP003882.1), *B. bavariensis* PBi (accession number NC_006156.1), *B. valaisiana* VS116 (accession number AB236666.1), *B. bissettiae* CA128 (accession number DQ393343), *B. lusitaniae* PoTiB1 (accession number DQ111035.1), *B. californiensis* CA446 (accession number DQ393347.1), *B. spielmanii* A14S (accession number ABKB02000003.1) and *B. sinica* CMN3 (accession number AB022138.1). These multiple sequence alignments supported the design of primers and TaqMan probes (labelled with fluorophores with different emission spectra), targeting the *B. burgdorferi* s.l. complex, and each of the four main species circulating in Europe (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae*). *Borrelia*-targeted primers and probes (Table 1) were designed using a combination of tools, including Primer Express 3.0 (Applied Biosystems) and BLAST (Altschul et al., 1997). Additional sets of primers and probes were also used as controls to detect ixodid 18S rDNA (Table 1), and the mammal β-actin-
coding-sequence (ACTB) (Costa et al., 2013), for assessment of PCR inhibition when DNA extracts from clinical samples or ixodid ticks were used as template.

Two-step multiplex real-time PCR

The algorithm used for the amplification-based screening of *B. burgdorferi* s.l. involves two steps (Figure 1). The first comprised a duplex real-time PCR, targeting both *flaB* gene (for the detection and quantification of *B. burgdorferi* s.l.), and an internal control (18S rDNA when using DNA extracts from adult ticks homogenates as template, or the *β-actin* gene when using DNA extracts from clinical samples). Samples for which positive amplification results were obtained (*flaB*/18S rDNA or *flaB*/ACTB), were further analyzed by a tetraplex real-time PCR specifically targeting the *flaB* gene of *B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. burgdorferi* s.s. (Figure 1. Amplification protocols were optimized using DNA extracted from pure cultures of *B. burgdorferi* s.l. as template.

Duplex real-time PCR reactions were carried out in a total volume of 20 μl using SensiFAST™ amplification mix (Bioline), 0.3 μM of each primer (*F_Bbsl*, *R_Bbsl*; *F_18S* rDNA, *R_18S* rDNA or *F_β-actin*, *R_β-actin*), 0.25 μM of each TaqMan probe (*P_Bbsl*; *P_18S* rDNA or *P_β-actin*), DNase-free water (Bioline), and 2 μl of extracted DNA template (corresponding to 20-40 ng of total DNA). The thermal cycling conditions were: 1 cycle at 95 °C for 1 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 45 s. Tetraplex real-time PCR reactions used SensiFAST™ amplification mix, 0.3 μM of *F_Bspp*, *R_Bspp* primers, and 0.25 μM of *P_Bafz*, *P_Bgar*, *P_Bbss* and 0.15 μM of *P_Blus TaqMan* probes in a total volume of 20 μl. The thermal cycling conditions were: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95 °C for 10 s and 60°C for 30 s. All samples with positive amplification results were retested for confirmation. Non-template
negative controls were included in each run to rule out the possibility of false-positive
results due to cross-contamination. Thermal cycling, fluorescent data collection, and data
analysis were performed in a 7500 Fast real-time PCR System (Applied Biosystems),
according to the manufacturer’s instructions.

**B. burgdorferi** s.l. reference strains

*B. burgdorferi* s.s. (B31), *B. afzelii* (PGau), *B. garinii* (PBi), *B. lusitaniae* (PoHL1), *B.
bavariensis* (PBi) and *B. valaisiana* (VS116) (maintained as part of the collection of
reference strains at the Leptospirosis and Lyme Borreliosis laboratory at IHMT/UNL),
were cultured in BSK-H medium at 34 ºC, and the growth was regularly followed by
observation under a dark-field microscope and evaluation of optical density (OD) by
spectrophotometric analysis (600 nm). When the cultures reached the exponential growth
phase (OD≈0.5), bacteria were harvested by centrifugation at 14,000×g, and their
genomic DNA extracted with the Gentra Puregene commercial kit (QIAGEN®),
according to the manufacturer’s instructions. DNA concentration and purity were
estimated using a NanoDrop 1000 spectrophotometer (NanoDrop™). The DNA
concentration was adjusted to 10⁶ GE for the six *B. burgdorferi* s.l. genospecies and
dilutions from 10⁶ to 10 GE were prepared.

Analytical specificity and sensitivity

To investigate whether the designed probes and respective flanking primers were able to
specifically detect their targets, PCR amplifications were carried out using as template
DNA from *B. burgdorferi* s.l., and also DNA templates extracted from three other
spirochetes (*Borrelia miyamotoi, Leptospira interrogans* and *Treponema pallidum*), and
from tick-borne piroplasms (Theileria sp. and Babesia sp.; Step A1 in Supplementary Figure 1).

To estimate the detection threshold (analytical sensitivity) of the duplex real-time PCR (involving partial amplification of flaB sequences plus an internal control) the template used corresponded to genomic DNA extracted from pure cultures of B. burgdorferi s.s., B. garinii, B. afzelii, B. lusitaniae, B. bavariensis and B. valaisiana. Likewise, the evaluation of the sensitivity of the tetraplex real-time PCR step (exclusively involving partial amplification of flaB) was carried out using extracts of B. afzelii, B. garinii, B. lusitaniae and B. burgdorferi s.s. genomic DNA. For each PCR step (duplex and tetraplex) a standard curve was constructed using 10-fold serial dilutions of the prepared DNA extracts, tested either individually or as mixtures of each of the templates used (in equivalent amounts; Step B in Supplementary Figure 1). These dilutions ranged from $10^6$ to 10 GE (10 GE = 50 fg of DNA), as defined by the National Reference Centre for Borrelia (NRZ units) and according to Rijpkema et al., 1997. PCR assays were performed in triplicate. The end-point corresponded to the last dilution for which the assay could still detect the respective DNA targets in all three replicates.

To ascertain whether the real-time PCR assays could be applied to the analysis of clinical samples, aliquots of human sera were spiked with a 10-fold serial dilution of B. burgdorferi s.l. DNA, using equivalent amounts of DNA from B. burgdorferi s.s., B. afzelii, B. garinii and B. lusitaniae, ranging from $10^6$ to 10 of GE (Step C in Supplementary Figure 1). Total DNA was re-extracted from this mixture using the Gentra Puregène commercial kit (QIAGEN®), according to the manufacturer’s instructions. Experimentally spiked samples were screened by two conventional PCR (Rijpkema et al., 1995; Wodecka et al., 2010), as well as the two real-time PCR assays here described.
Evaluation of real-time PCR with field-collected ticks and clinical samples

A panel of human samples (Table 2), was used to assess the performance of the two-step real-time PCR assay. The panel included samples of sera (n=20) and CSF (n=10) collected between 2012 and 2015 by the Leptospirosis and Lyme Borreliosis Group (IHMT/UNL), that having been anonymized prior to testing (Step D in Supplementary Figure 1). Also samples of questing nymphs and adult specimens of *Ixodes ricinus* (n=50), collected across Portugal in former studies (Nunes et al., 2015; Nunes et al., 2016; Step D in Supplementary Figure 1), were analyzed. The presence of *B. burgdorferi* s.l. DNA in all these samples had been formerly evaluated by two nested-PCR assays targeting the 23S-5S intergenic spacer region (Rijpkema et al., 1995) and *flaB* (Wodecka et al., 2010). Nested-PCR amplification products from tick samples had also been previously sequenced for the identification of *B. burgdorferi* s.l. genospecies. The entire study was carried out in strict accordance with protocols approved by the Institute of Hygiene and Tropical Medicine Ethics Committee, and the Portuguese Data Protection Authority.

Statistical analysis

For measuring the agreement between the results of the routinely performed molecular identification of clinical and tick samples, and the real-time PCR assay, kappa coefficient (k) was used. This coefficient, with confidence intervals, was determined with BioEstat 5.0.
Results

Analytical specificity and sensitivity

The real-time PCR assays optimized in the course of this work (duplex + tetraplex) allowed detecting *B. burgdorferi* s.l. DNA with high specificity as unspecific amplification products were not detected when DNA extracts from *B. miyamotoi*, *T. pallidum*, *L. interrogans*, *Theileria* sp. or *Babesia* sp. were used as template in repeated experiments (Step A1 in Supplementary Figure 1). In addition, no false positive results were ever detected when the four *Borrelia* species-specific probes were tested using as template DNA extracts of *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae.* TaqMan probes designed were shown to be specific for their respective targets.

For the evaluation of the sensitivity of the assay, *flaB* sequences were tentatively detected in serial dilutions (from $10^6$ to 10 GE) of extracts of DNA prepared from pure cultures of *B. burgdorferi* s.l. (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. lusitaniae*, *B. bavariensis*, and *B. valaisiana*). For the duplex real-time PCR (aiming at *B. burgdorferi* s.l. detection) *flaB* amplification was carried out either using each individual DNA extract *per se*, or an equivalent mixture of all of them (serially diluted to 10 GE). The sensitivity of the tetraplex real-time PCR was evaluated using a similar approach, i.e. with dilutions of DNA from *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. lusitaniae*, tested individually and as a mixture (Step B in Supplementary Figure 1).

The first duplex reaction could detect the presence of *B. burgdorferi* s.l. down to 100 fg of template DNA (equivalent to 20 GE; Figure 3A), regardless of the genospecies tested (Step B1 in Supplementary Figure 1). The standard curve for DNA mixture showed a correlation coefficient ($R^2$) of 0.98 and a slope of −3.2, corresponding to an efficiency of 105.35% (Figure 2B). For the tetraplex reaction, when each probe was tested individually,
the detection limit was 100 fg = 20 GE for *B. afzelii* (Ct ≈ 37), *B. garinii* (Ct ≈ 37) and *B. lusitaniae* (Ct ≈ 37) and 1 pg = 2×10^2 GE for *B. burgdorferi* s.s. (Ct ≈ 36), (a representative example for *B. lusitaniae* is shown in Figure 3A); when tested in tetraplex, the detection limit was 1 pg = 2×10^2 GE for *B. afzelii* (Ct ≈ 32), *B. garinii* (Ct ≈ 35), *B. lusitaniae* (Ct ≈ 35) and *B. burgdorferi* s.s. (Ct ≈ 35), a representative example for 2×10^6 GE is shown in Figure 3B; Step B1 in Supplementary Figure 1). The standard curves for the tetraplex reaction showed correlation coefficients (R^2) ranging from 0.929 to 0.997 and slopes of -2.296 to -3.377 (Figure 3C).

Experimentally spiked serum samples

A series of DNA extracts purified from human sera samples spiked with *B. burgdorferi* s.l. DNA was screened using the optimized duplex and tetraplex real-time PCR assays. The duplex assay could detect down to 20 GE of *Borrelia* DNA, with a Ct value of 35 (Step C1 in Supplementary Figure 1). The tetraplex assay, could detect 2×10^2 GE for each of the four genospecies tested, to which corresponded Ct values of 32 for *B. afzelii* and *B. burgdorferi* s.s., 35 for *B. garinii*, and 34 for *B. lusitaniae* (Step C1 Supplementary Figure 1).

Field-collected ticks and clinical samples

From the 50 tick samples tested, 24 were previously positive using the two conventional nested-PCR assays (see Materials and methods) for the presence of *B. burgdorferi* s.l. DNA. Furthermore, these tick samples were also tested by the two real-time PCR protocols, being 24 samples positive by the duplex real-time PCR (100%, test k = 1.000), and 23 by the tetraplex real-time PCR, (96%, test k = 0.960) (Table 2). The genospecies
of *B. burgdorferi* s.l. identified by the tetraplex were in agreement with those previously defined by DNA sequencing (Table 2). Regarding the 26 tick samples for which the nested-PCRs could not detect the presence of *Borrelia* DNA, the real-time PCR approaches also confirmed the apparent absence of *Borrelia* in these samples.

A similar algorithm was used for the analysis of the clinical samples (*n*=30), including sera and CSF, being 11 (37%, *n*=5 sera, *n*=6 CSF) positive by the nested-PCR protocols, for *Borrelia* DNA, and also by the duplex real-time PCR. However, only three of the samples (10% of the total of clinical samples analyzed) were positive using the tetraplex assay. The genospecies of *B. burgdorferi* s.l. identified included *B. afzelii;* *B. garinii* and *B. lusitaniae* (Table 2). The calculated k values were 0.920 for the duplex assay (taking into account the results obtained for sera and CSF samples), and 0.692 for the tetraplex assay, considering only the results obtained for the sera samples (since there were no positive results when CSF samples were tested).

**Discussion**

According to the European Center for Disease Prevention and Control (ECDC, 2016), the diagnosis of infections caused by *Borrelia* spp should be based on the identification of clinical symptoms, on the analysis of the patient’s medical history (evaluation of the risk of exposure to infected ticks), along with laboratory testing for detection of IgM/IgG specific antibodies (Bil-Lula et al., 2015). However, the serologic tests used for detection of an immune response against these bacteria give rise to large numbers of false negative results, most probably due to the kinetics of IgM production, which are absent during the so-called “window period”. Consequently, the development of molecular approaches for detection of these bacteria, and especially those based on real-time PCR, would be helpful for testing early-onsets of disease, before an antibody response develops. These tools
would also prove valuable for laboratory diagnosis of infections caused by *Borrelia* spp.
in biological samples collected from patients presenting non-classic symptoms.

Different *Borrelia* genospecies are associated with diverse hosts (Mannelli et al., 2012),
different clinical presentations (van Dam et al., 1993), severity of disease (Jungnick et al.,
2015), and geographic distribution (Stanek & Strle, 2003). Consequently, it is
increasingly important to detect and identify the diverse *Borrelia* genospecies involved
in any given infection.

This work describes, for the first time, the development of a tetraplex PCR protocol for
detection/quantification of four of the most prevalent *Borrelia* genospecies in Europe.
The assay in question corresponds to a combined multiplex *TaqMan* real-time PCR
strategy to infer the presence of *B. burgdorferi* s.l. genospecies in both clinical and vector
samples. In a first step, the presence of *Borrelia* spp is revealed by targeting *flaB* gene,
while an internal control is used, to monitor PCR inhibitions, targeting either tick or
human sequences, depending on the type of specimen under analysis. The second step of
the assay allows the simultaneous detection/quantification of four of the most prevalent
genospecies of *B. burgdorferi* s.l. not only in Portugal but also in Europe. Although both
amplification steps target the same gene, the primers used in the second step were
designed so as to allow the amplification of a distinct, more variable region of *flaB*, with
sufficient polymorphisms to allow their differential detection with species-specific
*TaqMan* probes.

In the duplex real-time PCR, DNA from each *B. burgdorferi* s.l. genospecies, whether
tested individually or simultaneously, a sensitivity of 20 GE was obtained, matching those
achieved with previously reported detection methods (Gooskens et al., 2006; O’Rourke
et al., 2013; Venczel et al., 2016). Furthermore, the assay also revealed high specificity,
as it failed to detect non-*Borrelia burgdorferi* s.l. *flaB* sequences. On the other hand, the
second amplification reaction was carried out in a tetraplex format that allowed the identification of *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, and *B. lusitaniae* with slight lower sensitivity (200 GE) most probably due to probe competition for their targets. When testing DNA extracts prepared from field-collected ticks, the first detection step displayed equivalent high performance when compared with the results obtained with the pure cultures of *B. burgdorferi* s.l., with one single exception, the same holds true for the second step of the assay (tetraplex format). Only one of the samples previously defined as positive for the presence of *Borrelia* spp. DNA yielded a negative result, probably due to the lower detection limit of the assay when dealing with the detection of *B. garinii*, *B. afzelii*, or *B. lusitaniae*. In all the other situations, the *B. burgdorferi* s.l. genospecies identified by the tetraplex assay were 100% concordant with previous identifications based on DNA sequence analysis.

Since Lyme borreliosis is not considered endemic in Portugal the consequent number of cases reported annually is small, a situation that is still negatively affected by scarce knowledge of physicians about LB. In any case, although the number of clinical samples available for analysis in this study was limited, the assessment of the sensitivity/specificity of the real-time PCR protocols, was further extended to the analysis of human serum and CSF samples. While the duplex assay disclosed 100% agreement with results previously obtained based on nested-PCR protocols followed by DNA sequencing, only three samples revealed the presence of DNA from *Borrelia* spp with the tetraplex assay. The remainder eight nested-PCR-positive samples were found negative by the tetraplex assay, probably conditioned by a loss of sensitivity when the four probes are used simultaneously with DNA extracted from these samples, and especially from CSF samples.
Previously published studies showed that *Borrelia* counts in CSF are very low (Nocton et al., 1996; Schwaiger et al., 2001; Gooskens et al., 2006; Bil-Lula et al., 2015), further compromising the detection of *Borrelia* DNA. Since the method here described makes use of the inclusion of internal controls in each PCR run, the possibility of low-test sensitivity due to the presence of PCR inhibitors in CSF samples can be excluded. Moreover, this decrease of sensitivity is characteristic of multiplex assays in general when clinical samples are tested, and not a feature of the specific tetraplex qPCR assay here reported.

In any case, and despite the assay's lower performance using CSF, both real-time PCR protocols were developed to be used at the beginning of the infection, so as to improve early diagnosis of LB, at a moment where detection of *Borrelia* should not rely on the use of CSF samples, being their analysis especially valuable in an advance stages of the infection (chronic phase).

In conclusion, this two-step multiplex *TaqMan* real-time PCR assay targeting the *flaB* locus, proved to be an efficient method especially when screening for *Borrelia* infection in tick samples, and a promising tool for early diagnosis purposes on clinical serum samples. Moreover, the ability to detect four of the most prevalent *B. burgdorferi* s.l. genospecies in Europe in a single-run has both time-saving and cost-reduction added value when compared with the conventional PCR and sequencing methods.

**Acknowledgments**

This work was supported by Ministry of Education and Science of Portugal, Fundação para a Ciência e a Tecnologia, through a PhD grant (SFRH/BD/78325/2011), and Funds from GHTM – UID/Multi/04413/2013.
References


Tables and respective legends:

Table 1 – Sequences of primers and probes designed for this study.

<table>
<thead>
<tr>
<th>Primers/Probes</th>
<th>Primer/Probe sequence (5’-3’)</th>
<th>Complementary target</th>
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<tr>
<td><strong>F_Bbsl</strong></td>
<td>TTAATGGTCCTACCACTGCTTTGTTGA</td>
<td><em>flaB</em> gene of <em>B. burgdorferi</em> s.l. complex</td>
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<td><strong>F_B-actin</strong></td>
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<td><em>β-actin</em> gene of mammals</td>
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<tr>
<td><strong>P_18S rRNA</strong></td>
<td>FAM – CGGGTGCTTATTAGGACAGAT – BHQ1</td>
<td></td>
</tr>
</tbody>
</table>

*Bbsl* – *Borrelia burgdorferi* sensu lato; *Bafz* – *Borrelia afzelii*; *Bgar* – *Borrelia garinii*; *Blus* – *Borrelia lusitaniae*; *Bbs* – *Borrelia burgdorferi* sensu stricto; 1 Probe labeled with FAM (carboxyfluorescein) fluorophore and BHQ1 quencher; 2 Probe labeled with ROX (6-carboxyX-rhodamine) fluorophore and BHQ2 quencher; 3 Probe labeled with Cy5 (Cyanine) fluorophore and BHQ2 quencher; 4 Probe labeled with JOE fluorophore and BHQ1 quencher.
Table 2 – Comparison of duplex and tetraplex real-time PCR’s positive samples with results from previous sequencing for tick samples.

<table>
<thead>
<tr>
<th>Samples (nested-PCR’s positive or negative)</th>
<th>Sequencing results</th>
<th>Duplex real-time PCR</th>
<th>Tetraplex real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, <em>B. afzelii</em></td>
<td>1 positive (Ct ≈ 17)</td>
<td>1 <em>B. afzelii</em> (Ct ≈ 21)</td>
<td></td>
</tr>
<tr>
<td>3, <em>B. burgdorferi</em> s.s.</td>
<td>3 positive</td>
<td>2 <em>B. burgdorferi</em> s.s. (Ct ≈ 33; Ct ≈ 36)</td>
<td></td>
</tr>
<tr>
<td>8, <em>B. garinii</em></td>
<td>8 positive (Ct ≈ 17 to Ct ≈ 19)</td>
<td>8 <em>B. garinii</em> (Ct ≈ 16 to Ct ≈ 33)</td>
<td></td>
</tr>
<tr>
<td>12, <em>B. lusitaniae</em></td>
<td>12 positive (Ct ≈ 18 to Ct ≈ 26)</td>
<td>12 <em>B. lusitaniae</em> (Ct ≈ 20 to Ct ≈ 35)</td>
<td></td>
</tr>
<tr>
<td>26 negative</td>
<td>--------</td>
<td>26 negative</td>
<td></td>
</tr>
</tbody>
</table>

Clinical samples (n= 30)

<table>
<thead>
<tr>
<th>Samples (nested-PCR’s positive or negative)</th>
<th>Sequencing results</th>
<th>Duplex real-time PCR</th>
<th>Tetraplex real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 sera</td>
<td>--------</td>
<td>1 serum as <em>B. afzelii</em> (Ct ≈ 29)</td>
<td></td>
</tr>
<tr>
<td>6 CSF (positive)</td>
<td>--------</td>
<td>1 serum as <em>B. garinii</em> (Ct ≈ 30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 serum as <em>B. lusitaniae</em> (Ct ≈ 32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 negative (2 sera; 6 CSF)</td>
<td></td>
</tr>
<tr>
<td>15 sera; 4 CSF (negative)</td>
<td>--------</td>
<td>19 negative</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19 negative</td>
<td></td>
</tr>
</tbody>
</table>
**Figures legends:**

**Figure 1** - Representation of the real-time PCR algorithm for identification/quantification of *B. burgdorferi* s.l. genospecies. The targeted genes are indicated above the arrows (B. – *Borrelia*; Bafz – *B. afzelii*; Bgar – *B. garinii*; Blus – *B. lusitaniae*; Bbss – *B. burgdorferi* s.s.).
**Figure 2** – Illustration of the duplex real-time PCR amplification curve obtained for each *Borrelia burgdorferi* s.l. genospecies as a function of the DNA concentration (expressed as GE) used (A), and respective linear relationship between the logarithm of the starting concentration of DNA and the amplification Ct values (B). Neg-real-time PCR negative control, using DNase free water as template, Ct-interception in the minimum threshold (20 GE), RFU-Relative Fluorescence Units.
Figure 3 - Illustration of the tetraplex real-time PCR amplification curves obtained for each probe as a function of the DNA concentration used. Similar results were obtained for all the species/probes tested, and only those obtained for *B. lusitaniae* are shown (A). The graph in (B) shows the results obtained when a mixture of the four DNA templates (*B. lusitaniae*, *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*) and their specific probes were used in a tetraplex format. The respective linear relationship between the logarithm of the starting concentration of DNA and the amplification Ct values is indicated in (C). Neg-real-time PCR negative control using DNase free water as template; Ct-interception in the minimum threshold ($2\times10^2$ GE for the four genospecies); RFU-Relative Fluorescence Units.
Supplementary Figure 1 – Flowchart with the several steps developed for the optimization and evaluation of the real-time PCR algorithm for B. burgdorferi s.l. genospecies.