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

Mónica Nunes[1,2], Ricardo Parreira[2], Teresa Carreira[2], João Inácio[2,3], Maria Luísa Vieira[2]

[1]iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal;

[2]Unidade de Microbiologia Médica, Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL, Rua da Junqueira nº100, 1349-008 Lisboa, Portugal;

[3]School of Pharmacy and Biomolecular Sciences, University of Brighton, Lewes road, Brighton BN2 4GJ, United Kingdom;

Correspondence should be addressed to:

Mónica Nunes

iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal;

(E-mail: monicanunes@ihmt.unl.pt)

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.

51 **Keywords:** Lyme borreliosis, molecular diagnosis, multiplex *TaqMan* real-time PCR,
52 *Borrelia burgdorferi* s.l. species differentiation

53

54 ## Introduction

55 Lyme borreliosis (LB) is known as the most common vector-borne disease in both Europe
56 and North America (ECDC, 2016). The number of cases in the last two decades points to
57 360 000 cases in Europe (with a marked increase) (ECDC, 2014), and approximately
58 300 000 cases in the USA, between 1995 and 2015 (Lindgren & Jaenson, 2006; Hinckley
59 et al., 2014). Nearly all human cases in Europe are caused by three members of the *B.*
60 *burgdorferi* sensu lato (s.l.) complex, namely *B. garinii*, *B. afzelii,* and *B. burgdorferi*
61 sensu stricto (s.s.) (Rizzoli et al., 2011), all of which are transmitted through the bite of
62 *Ixodes ricinus* ticks. In the USA, *B. burgdorferi* s.s. was the only species associated to
63 LB. However, more recently *Candidatus* B. mayonii was also identified as the causative
64 agent of LB (Stanek et al., 2012, Pritt et al., 2016).

65 The clinical manifestations of LB are wide-ranging, and linked with differential bacterial
66 tropisms for distinct tissues or systems (van Dam et al., 1993; Balmelli & Piffaretti, 1995).
67 Early localized infections typically result in a rash known as erythema migrans (EM),
68 from which spirochetes can disseminate to the central and peripheral nervous systems,
69 joints, and other organs. Infections with *B. burgdorferi* s.s. are usually associated with
70 arthritis, while those caused by *B. garinii* and *B. afzelii* are usually accompanied by
71 neurological and skin complications (e.g. Bell's palsy, encephalopathy and acrodermatitis
72 chronica atrophicans – ACA), respectively (van Dam et al., 1993). Nevertheless, LB may
73 also remain latent, without an unequivocal clinical presentation, or translate into a clinical
74 presentation including unspecific symptoms such as headache, myalgia, arthralgia or
75 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).

99 The aim of this study was to develop an easy-to-use *TaqMan* real-time PCR assay for the
100 detection of *B. burgdorferi* s.l. spirochetes, also allowing the differentiation of clinically-
101 relevant species of the complex.

102

103 **Material and methods**

104 TaqMan probes and flanking primers

105 A multiple sequence alignment of *flaB* [located in the bacterial linear chromosome,
106 which encodes a 41-kDa flagellin protein (Wang,1999)] reference sequences retrieved
107 from GenBank was created using Mafft 7 (Katoh Standley, 2013). The flagellin-coding
108 sequences used included those of *B. burgdorferi* s.s. B31 (accession number
109 CP009656.1), *B. garinii* SZ (accession number CP007564.1), *B. afzelii* HLJ01 (accession
110 number CP003882.1), *B. bavariensis* PBi (accession number NC_006156.1), *B.*
111 *valaisiana* VS116 (accession number AB236666.1), *B. bissettiae* CA128 (accession
112 number DQ393343), *B. lusitaniae* PoTiB1 (accession number DQ111035.1), *B.*
113 *californiensis* CA446 (accession number DQ393347.1), *B. spielmanii* A14S (accession
114 number ABKB020000003.1) and *B. sinica* CMN3 (accession number AB022138.1).
115 These multiple sequence alignments supported the design of primers and *TaqMan* probes
116 (labelled with fluorophores with different emission spectra), targeting the *B. burgdorferi*
117 s.l. complex, and each of the four main species circulating in Europe (*B. burgdorferi* s.s.,
118 *B. afzelii*, *B. garinii* and *B. lusitaniae*). *Borrelia*-targeted primers and probes (Table 1)
119 were designed using a combination of tools, including Primer Express 3.0 (Applied
120 Biosystems) and BLAST (Altschul et al., 1997). Additional sets of primers and probes
121 were also used as controls to detect ixodid 18S rDNA (Table 1), and the mammal *β-actin-*

122 coding-sequence (ACTB) (Costa et al., 2013), for assessment of PCR inhibition when
123 DNA extracts from clinical samples or ixodid ticks were used as template.

124

125 Two-step multiplex real-time PCR

126 The algorithm used for the amplification-based screening of *B. burgdorferi* s.l. involves
127 two steps (Figure 1). The first comprised a duplex real-time PCR, targeting both *flaB*
128 gene (for the detection and quantification of *B. burgdorferi* s.l.), and an internal control
129 (18S rDNA when using DNA extracts from adult ticks homogenates as template, or the
130 *β-actin* gene when using DNA extracts from clinical samples). Samples for which
131 positive amplification results were obtained (*flaB*/18S rDNA or *flaB*/ACTB), were further
132 analyzed by a tetraplex real-time PCR specifically targeting the *flaB* gene of *B. afzelii*, *B.*
133 *garinii*, *B. lusitaniae* and *B. burgdorferi* s.s. (Figure 1. Amplification protocols were
134 optimized using DNA extracted from pure cultures of *B. burgdorferi* s.l. as template.

135 Duplex real-time PCR reactions were carried out in a total volume of 20 µl using
136 SensiFAST™ amplification mix (Bioline), 0.3 µM of each primer (F_*Bb*sl, R_*Bb*sl;
137 F_18S rDNA, R_18S rDNA or F_*β-actin*, R_ *β-actin*), 0.25 µM of each *TaqMan* probe
138 (P_*Bb*sl; P_18S rDNA or P_ *β-actin*), DNase-free water (Bioline), and 2 µl of extracted
139 DNA template (corresponding to 20-40 ng of total DNA). The thermal cycling conditions
140 were: 1 cycle at 95 °C for 1 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for
141 45 s. Tetraplex real-time PCR reactions used SensiFAST™ amplification mix, 0.3 µM of
142 F_*B*spp, R_*B*spp primers, and 0.25 µM of P_*Bafz*, P_*Bgar*, P_*Bb*ss and 0.15 µM of
143 P_*Blus TaqMan* probes in a total volume of 20 µl. The thermal cycling conditions were:
144 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
145 samples with positive amplification results were retested for confirmation. Non-template

146 negative controls were included in each run to rule out the possibility of false-positive
147 results due to cross-contamination. Thermal cycling, fluorescent data collection, and data
148 analysis were performed in a 7500 Fast real-time PCR System (Applied Biosystems),
149 according to the manufacturer's instructions.

150

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

152 *B. burgdorferi* s.s. (B31), *B. afzelii* (PGau), *B. garinii* (PBi), *B. lusitaniae* (PoHL1), *B.*
153 *bavariensis* (PBi) and *B. valaisiana* (VS116) (maintained as part of the collection of
154 reference strains at the Leptospirosis and Lyme Borreliosis laboratory at IHMT/UNL),
155 were cultured in BSK-H medium at 34 ºC, and the growth was regularly followed by
156 observation under a dark-field microscope and evaluation of optical density (OD) by
157 spectrophotometric analysis (600 nm). When the cultures reached the exponential growth
158 phase (OD≈0.5), bacteria were harvested by centrifugation at 14,000×g, and their
159 genomic DNA extracted with the Gentra Puregene commercial kit (QIAGEN®),
160 according to the manufacturer's instructions. DNA concentration and purity were
161 estimated using a NanoDrop 1000 spectrophotometer (NanoDrop™). The DNA
162 concentration was adjusted to 10^6 GE for the six *B. burgdorferi* s.l. genospecies and
163 dilutions from 10^6 to 10 GE were prepared.

164

165 Analytical specificity and sensitivity

166 To investigate whether the designed probes and respective flanking primers were able to
167 specifically detect their targets, PCR amplifications were carried out using as template
168 DNA from *B. burgdorferi* s.l., and also DNA templates extracted from three other
169 spirochetes (*Borrelia miyamotoi*, *Leptospira interrogans* and *Treponema pallidum*), and

170 from tick-borne piroplasms (*Theileria* sp. and *Babesia* sp.; Step A1 in Supplementary
171 Figure 1).

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

186 To ascertain whether the real-time PCR assays could be applied to the analysis of clinical
187 samples, aliquots of human sera were spiked with a 10-fold serial dilution of *B.*
188 *burgdorferi* s.l. DNA, using equivalent amounts of DNA from *B. burgdorferi* s.s., *B*
189 *afzelii*, *B. garinii* and *B. lusitaniae*, ranging from 10^6 to 10 of GE (Step C in
190 Supplementary Figure 1). Total DNA was re-extracted from this mixture using the Gentra
191 Puregene commercial kit (QIAGEN®), according to the manufacturer's instructions.
192 Experimentally spiked samples were screened by two conventional PCR (Rijpkema et al.,
193 1995; Wodecka et al., 2010), as well as the two real-time PCR assays here described.

194

195 Evaluation of real-time PCR with field-collected ticks and clinical samples

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

210

211 Statistical analysis

212 For measuring the agreement between the results of the routinely performed molecular
213 identification of clinical and tick samples, and the real-time PCR assay, kappa coefficient
214 (k) was used. This coefficient, with confidence intervals, was determined with BioEstat
215 5.0.

216

217

218 **Results**

219 Analytical specificity and sensitivity

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

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

237 The first duplex reaction could detect the presence of *B. burgdorferi* s.l. down to 100 fg
238 of template DNA (equivalent to 20 GE; Figure 3A), regardless of the genospecies tested
239 (Step B1 in Supplementary Figure 1). The standard curve for DNA mixture showed a
240 correlation coefficient (R^2) of 0.98 and a slope of – 3.2, corresponding to an efficiency of
241 105,35% (Figure 2B). For the tetraplex reaction, when each probe was tested individually,

242 the detection limit was 100 fg = 20 GE for *B. afzelii* (Ct ≈ 37), *B. garinii* (Ct ≈ 37) and *B.*
243 *lusitaniae* (Ct ≈ 37) and 1 pg = $2{\times}10^2$ GE for *B. burgdorferi* s.s. (Ct ≈ 36), (a
244 representative example for *B. lusitaniae* is shown in Figure 3A); when tested in tetraplex,
245 the detection limit was 1 pg = $2{\times}10^2$ GE for *B. afzelii* (Ct ≈ 32), *B. garinii* (Ct ≈ 35), *B.*
246 *lusitaniae* (Ct ≈ 35) and *B. burgdorferi* s.s. (Ct ≈ 35), a representative example for $2{\times}10^6$
247 GE is shown in Figure 3B; Step B1 in Supplementary Figure 1). The standard curves for
248 the tetraplex reaction showed correlation coefficients (R^2) ranging from 0.929 to 0.997
249 and slopes of -2.296 to -3.377 (Figure 3C).

250

251 Experimentally spiked serum samples

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

259

260 Field-collected ticks and clinical samples

261 From the 50 tick samples tested, 24 were previously positive using the two conventional
262 nested-PCR assays (see Materials and methods) for the presence of *B. burgdorferi* s.l.
263 DNA. Furthermore, these tick samples were also tested by the two real-time PCR
264 protocols, being 24 samples positive by the duplex real-time PCR (100%, test k = 1.000),
265 and 23 by the tetraplex real-time PCR, (96%, test k = 0.960) (Table 2). The genospecies

266 of *B. burgdorferi* s.l. identified by the tetraplex were in agreement with those previously
267 defined by DNA sequencing (Table 2). Regarding the 26 tick samples for which the
268 nested-PCRs could not detect the presence of *Borrelia* DNA, the real-time PCR
269 approaches also confirmed the apparent absence of *Borrelia* in these samples.
270 A similar algorithm was used for the analysis of the clinical samples (n=30), including
271 sera and CSF, being 11 (37%, n=5 sera, n=6 CSF) positive by the nested-PCR protocols,
272 for *Borrelia* DNA, and also by the duplex real-time PCR. However, only three of the
273 samples (10% of the total of clinical samples analyzed) were positive using the tetraplex
274 assay. The genospecies of *B. burgdorferi* s.l. identified included *B. afzelii*; *B. garinii* and
275 *B. lusitaniae* (Table 2). The calculated k values were 0.920 for the duplex assay (taking
276 into account the results obtained for sera and CSF samples), and 0.692 for the tetraplex
277 assay, considering only the results obtained for the sera samples (since there were no
278 positive results when CSF samples were tested).

279 **Discussion**

280 According to the European Center for Disease Prevention and Control (ECDC, 2016), the
281 diagnosis of infections caused by *Borrelia* spp should be based on the identification of
282 clinical symptoms, on the analysis of the patient's medical history (evaluation of the risk
283 of exposure to infected ticks), along with laboratory testing for detection of IgM/IgG
284 specific antibodies (Bil-Lula et al., 2015). However, the serologic tests used for detection
285 of an immune response against these bacteria give rise to large numbers of false negative
286 results, most probably due to the kinetics of IgM production, which are absent during the
287 so-called "window period". Consequently, the development of molecular approaches for
288 detection of these bacteria, and especially those based on real-time PCR, would be helpful
289 for testing early-onsets of disease, before an antibody response develops. These tools

290 would also prove valuable for laboratory diagnosis of infections caused by *Borrelia* spp.
291 in biological samples collected from patients presenting non-classic symptoms.

292 Different *Borrelia* genospecies are associated with diverse hosts (Mannelli et al., 2012),
293 different clinical presentations (van Dam et al., 1993), severity of disease (Jungnick et al.,
294 2015), and geographic distribution (Stanek & Strle, 2003). Consequently, it is
295 increasingly important to detect and identify the diverse *Borrelia* genospecies involved
296 in any given infection.

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

310 In the duplex real-time PCR, DNA from each *B. burgdorferi* s.l. genospecies, whether
311 tested individually or simultaneously, a sensitivity of 20 GE was obtained, matching those
312 achieved with previously reported detection methods (Gooskens et al., 2006; O'Rourke
313 et al., 2013; Venczel et al., 2016). Furthermore, the assay also revealed high specificity,
314 as it failed to detect non-*Borrelia burgdorferi* s.l. *flaB* sequences. On the other hand, the

315 second amplification reaction was carried out in a tetraplex format that allowed the
316 identification of *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, and *B. lusitaniae* with slight
317 lower sensitivity (200 GE) most probably due to probe competition for their targets.

318 When testing DNA extracts prepared from field-collected ticks, the first detection step
319 displayed equivalent high performance when compared with the results obtained with the
320 pure cultures of *B. burgdorferi* s.l., with one single exception, the same holds true for the
321 second step of the assay (tetraplex format). Only one of the samples previously defined
322 as positive for the presence of *Borrelia* spp. DNA yielded a negative result, probably due
323 to the lower detection limit of the assay when dealing with the detection of *B. garinii*, *B.*
324 *afzelii*, or *B. lusitaniae*. In all the other situations, the *B. burgdorferi* s.l. genospecies
325 identified by the tetraplex assay were 100% concordant with previous identifications
326 based on DNA sequence analysis.

327 Since Lyme borreliosis is not considered endemic in Portugal the consequent number of
328 cases reported annually is small, a situation that is still negatively affected by scarce
329 knowledge of physicians about LB. In any case, although the number of clinical samples
330 available for analysis in this study was limited, the assessment of the
331 sensitivity/specificity of the real-time PCR protocols, was further extended to the analysis
332 of human serum and CSF samples. While the duplex assay disclosed 100% agreement
333 with results previously obtained based on nested-PCR protocols followed by DNA
334 sequencing, only three samples revealed the presence of DNA from *Borrelia* spp with the
335 tetraplex assay. The remainder eight nested-PCR-positive samples were found negative
336 by the tetraplex assay, probably conditioned by a loss of sensitivity when the four probes
337 are used simultaneously with DNA extracted from these samples, and especially from
338 CSF samples.

339 Previously published studies showed that *Borrelia* counts in CSF are very low (Nocton
340 et al., 1996; Schwaiger et al., 2001; Gooskens et al., 2006; Bil-Lula et al., 2015), further
341 compromising the detection of *Borrelia* DNA. Since the method here described makes
342 use of the inclusion of internal controls in each PCR run, the possibility of low-test
343 sensitivity due to the presence of PCR inhibitors in CSF samples can be excluded.
344 Moreover, this decrease of sensitivity is characteristic of multiplex assays in general when
345 clinical samples are tested, and not a feature of the specific tetraplex qPCR assay here
346 reported.

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

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

358

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363

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463 *Tables and respective legends:*

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

Primers/Probes	Primer/Probe sequence (5'- 3')	Complementary target
F_*Bb*sl	TTAATGTTACAACYACAGTTGA	*flaB* gene of *B. burgdorferi* s.l. complex
R_*Bb*sl	GCTACAATGACAGATGAGGT	
P_*Bb*sl[1]	JOE-AAGAGCAAATTTAGGTGCTTTCCAA – BHQ1	
***F_B*spp**	CAAGATGAAGCDATTGCTGTAAA	*flaB* gene of *B. afzelli, B. garinii, B. lusitaniae, B. burgdorferi* s.s.
***R_B*spp**	CTGCTACAGCACCTTCTCA	
P_*Bafz*[2]	ROX – TTCTTGAGCACCCTCTTGAACAGG – BHQ2	
P_*Bgar*[3]	Cy5 – CTTGTTGAGCTCCTTCTTGAACAGG – BHQ2	
P_*Blus*[4]	JOE – TTGAACACCTTCTTGAGCAGGTGCA – BHQ1	
P_*Bb*ss[1]	FAM –TCCTTCCTGTTGAACACCCTCTTG – BHQ1	
F_*B*-actin	GGCTCYATYCTGGCCTC	*β-actin* gene of mammals
F_*B*-actin	GCAYTTGCGGTGSACRATG	
P_*B*-actin[1]	FAM – TACTCCTGCTTGCTGATCCACATC – BHQ1	
F_18S rRNA	AGCTAATACATGCAGTGAGC	18S rRNA gene of ixodids
R_18S rRNA	TGATCGCATGGCCACGAG	
P_18S rRNA[1]	FAM – CGGGTGCTTTTATTAGACCAAGAT – BHQ1	

466 *Bb*sl – *Borrelia burgdorferi* sensu lato; *Bafz – Borrelia afzelii*; *Bgar – Borrelia garinii*; *Blus – Borrelia*
467 *lusitaniae*; *Bb*ss – *Borrelia burgdorferi* sensu stricto;[1] Probe labeled with FAM (carboxyfluorescein)
468 fluorophore and BHQ1 quencher; [2]Probe labeled with ROX (6-carboxyX-rhodamine) fluorophore and
469 BHQ2 quencher; [3]Probe labeled with Cy5 (Cyanine) fluorophore and BHQ2 quencher; [4]Probe labeled with
470 JOE fluorophore and BHQ1 quencher.

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475 **Table 2** – Comparison of duplex and tetraplex real-time PCR's positive samples with
476 results from previous sequencing for tick samples.

I. ricinus samples (n=50)			
Samples (nested-PCR's positive or negative)	**Sequencing results**	**Duplex real – time PCR**	**Tetraplex real-time PCR**
1	*B. afzelii*	1 positive (Ct ≈ 17)	1 *B. afzelii* (Ct ≈ 21)
3	*B. burgdorferi* s.s.	3 positive	2 *B. burgdorferi* s.s. (Ct ≈ 33; Ct ≈ 36) 1 negative
8	*B. garinii*	8 positive (Ct ≈ 17 to Ct ≈ 19)	8 *B. garinii* (Ct ≈ 16 to Ct ≈ 33)
12	*B. lusitaniae*	12 positive (Ct ≈ 18 to Ct ≈ 26)	12 *B. lusitaniae* (Ct ≈ 20 to Ct ≈ 35)
26 negative	-------	26 negative	26 negative
Clinical samples (n= 30)			
Samples (nested-PCR's positive or negative)	**Sequencing results**	**Duplex real – time PCR**	**Tetraplex real-time PCR**
5 sera 6 CSF (positive)	------- -------	11 positive (Ct ≈ 17 to Ct ≈ 36)	1 serum as *B. afzelii* (Ct ≈ 29) 1 serum as *B. garinii* (Ct ≈ 30) 1 serum as *B. lusitaniae* (Ct ≈ 32) 8 negative (2 sera; 6 CSF)
15 sera; 4 CSF (negative)	-------	19 negative	19 negative

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482 *Figures legends:*

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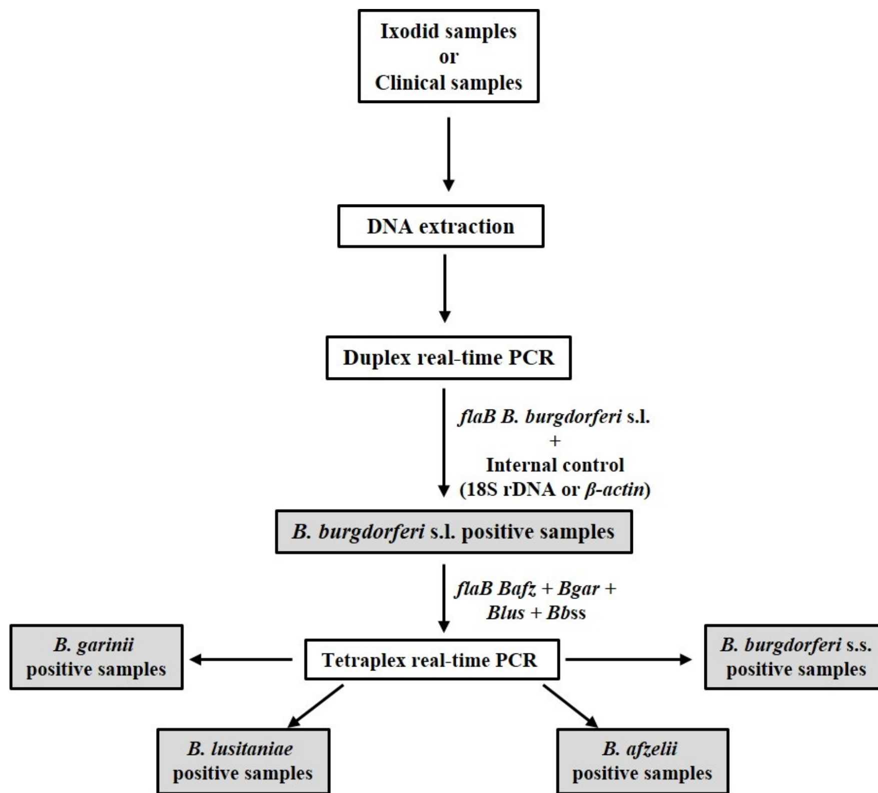

492 **Figure 1 -** Representation of the real-time PCR algorithm for identification/quantification
493 of *B. burgdorferi* s.l. genospecies. The targeted genes are indicated above the arrows (B.
494 – *Borrelia*; *Bafz* – *B. afzelii*; *Bgar* – *B. garinii*; *Blus* – *B. lusitaniae*; *Bb*ss – *B. burgdorferi*
495 s.s.).

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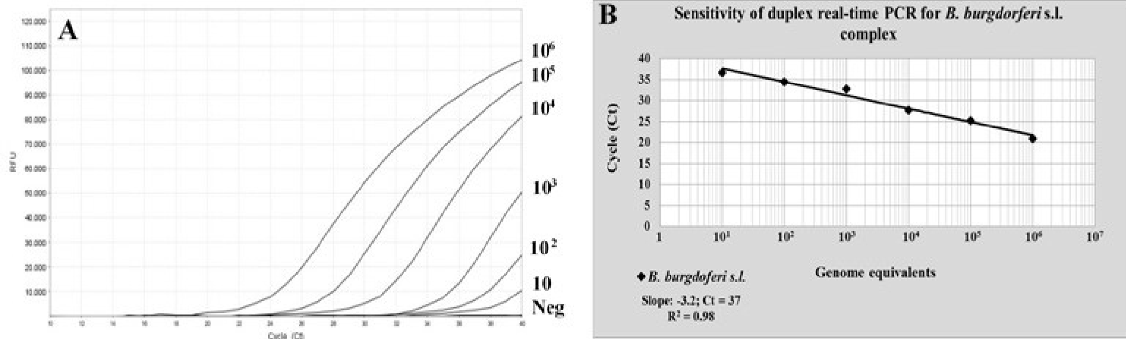
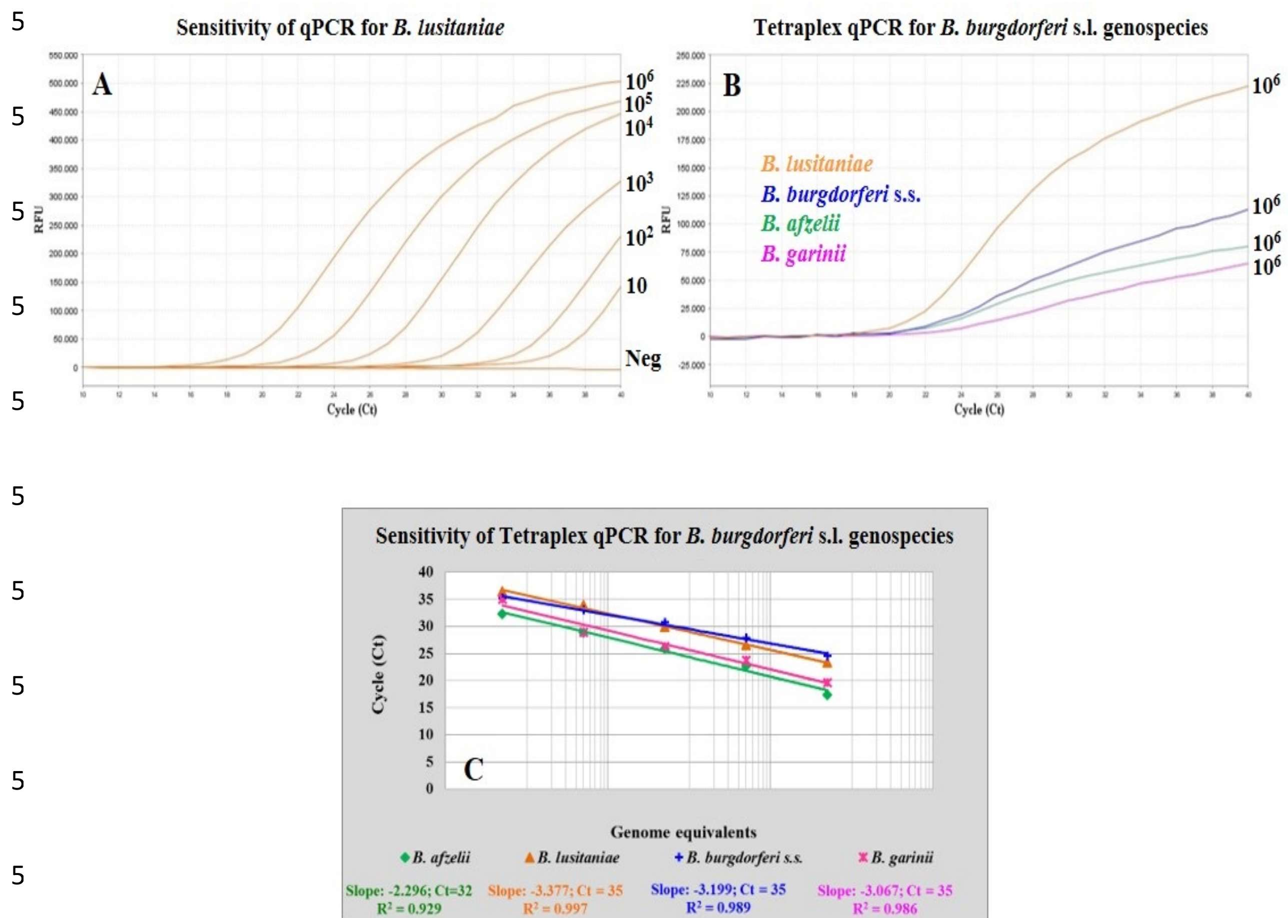


Figure 2 – Illustration of the duplex real-time PCR amplification curve obtained for each *Borrelia burgdorferi* s.l. genospecies as a function of the DNA concentration (expressed as GE) used (A), and respective linear relationship between the logarithm of the starting concentration of DNA and the amplification 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.



525 **Figure 3** - Illustration of the tetraplex real-time PCR amplification curves obtained for
526 each probe as a function of the DNA concentration used. Similar results were obtained
527 for all the species/probes tested, and only those obtained for *B. lusitaniae* are shown (A).
528 The graph in (B) shows the results obtained when a mixture of the four DNA templates
529 (*B. lusitaniae, B. burgdorferi* s.s., *B. afzelii* and *B. garinii*) and their specific probes were
530 used in a tetraplex format. The respective linear relationship between the logarithm of the
531 starting concentration of DNA and the amplification Ct values is indicated in (C). Neg-
532 real-time PCR negative control using DNase free water as template; Ct-interception in
533 the minimum threshold (2×10^2 GE for the four genospecies); RFU-Relative Fluorescence
534 Units.

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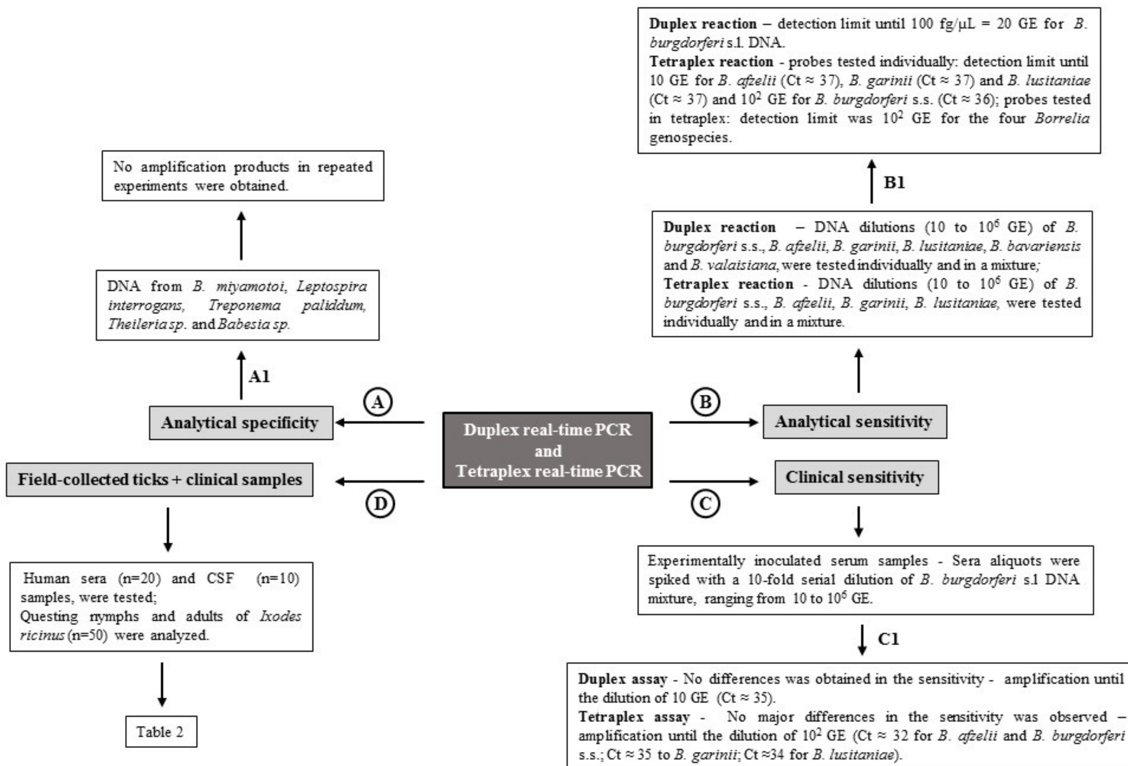

546 **Supplementary Figure 1** – Flowchart with the several steps developed for the
547 optimization and evaluation of the real-time PCR algorithm for *B. burgdorferi* s.l.
548 genospecies.