Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

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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ARTICLE INFO

Keywords:
Lyme borreliosis
Molecular diagnosis
Multiplex *TaqMan* real-time PCR
Borrelia burgdorferi s.l. species differentiation

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 has a different tropism that resuls 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 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.

1. 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 and 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 a 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 and 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

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http://dx.doi.org/10.1016/j.ttbdis.2017.09.001
Received 15 December 2016; Received in revised form 21 August 2017; Accepted 1 September 2017

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.

2. Material and methods

2.1. 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 and 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* C-A446 (accession number DQ393347.1), *B. spielmanii* A14S (accession number ABKB020000003.1) 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.

2.2. Two-step multiplex real-time PCR

The algorithm used for the amplification-based screening of *B. burgdorferi* s.l. involves two steps (Fig. 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. (Fig. 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_*Bb*sl, R_*Bb*sl; F_18S rDNA, R_18S rDNA or F_*β-actin*, R_ *β-actin*), 0.25 µM of each *TaqMan* probe (P_*Bb*sl; 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_*B*spp, R_*B*spp primers, and 0.25 µM of P_*Bafz*, P_*Bgar*, P_*Bb*ss 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.

2.3. 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 °C, and their 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 10^6 GE for the six *B. burgdorferi* s.l. genospecies and dilutions from 10^6 to 10 GE were prepared.

2.4. 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 Fig. 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