Ticks and Tick-borne Diseases 5 (2014) 722–726

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Borrelia spirochetes in Russia: Genospecies differentiation by real-time PCR

T.A. Mukhacheva, S.Y. Kovalev*

Laboratory of Molecular Genetics, Department of Biology, Ural Federal University, Lenin Avenue 51, Yekaterinburg 620000, Russia

ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 19 April 2014
Accepted 14 May 2014
Available online 7 August 2014

Keywords:
Borrelia
Genospecies
Mixed infection
Real-time PCR
Ixodes
Multilocus sequence typing

ABSTRACT

Spirochetes of the *Borrelia burgdorferi* sensu lato complex are the causative agent of Lyme borreliosis which is widespread in Russia. Nowadays, three clinically important *B. burgdorferi* s.l. genospecies, *B. afzelii*, *B. garinii*, *B. bavariensis* sp. nov., can be found in Russia, as well as *B. miyamotoi*, which belongs to the tick-borne relapsing fever group of spirochetes. Several techniques have been developed to differentiate *Borrelia* genospecies. However, most of them do not allow detection of all of these genospecies simultaneously. Also, no method based on the RT-PCR TaqMan approach has been proposed to differentiate the genetically closely related species *B. bavariensis* and *B. garinii*. In the present paper, we investigated two species of ticks, *I. persulcatus* and *I. pavlovskyi* (1343 and 92 adults, respectively). Two sets of primers and probes for RT-PCR, with uvrA, glpQ and nifS genes as targets, were designed to detect four *Borrelia* genospecies in positive samples. The average prevalence of *Borrelia* sp. was about 40%, with *B. afzelii* as the most prevalent genospecies. Mixed infections of *B. bavariensis* and *B. garinii* were found to be extremely rare. While *B. bavariensis* was predominant in *I. persulcatus*, *I. pavlovskyi* ticks were infected exclusively by *B. garinii*. The proposed technique proved to be efficient in selection of individual *Borrelia* species for further genetic analysis, in particular, for multilocus sequence typing. Also, it could be applied for the differentiation of *Borrelia* genospecies in clinical material.

Introduction

Spirochetes of the *Borrelia burgdorferi* sensu lato complex are the causative agent of Lyme borreliosis (LB), a multisystem zoonotic disease. LB is registered in 72 of the 85 regions of Russia with an annual incidence of 6400 to 9900 cases (Yastrebov et al., 2012). Until recently, two dominant *B. burgdorferi* s.l. genospecies were found in Russia, *B. afzelii* and *B. garinii*, with ixodid ticks as vectors, namely *Ixodes persulcatus* Schulze, *I. ricinus* L., and *I. pavlovskyi* Pomerantsev. Later, a new genospecies, *B. miyamotoi*, belonging to the tick-borne relapsing fever (TBRF) complex, was detected (Fomenko et al., 2008). A promising new technique, multilocus sequence typing (MLST) (Margos et al., 2008), allows division of *B. garinii* into two genospecies: *B. garinii* (hereinafter *B. garinii* s.s., which includes genogroups 20047 and ChY13p), and *B. bavariensis* sp. nov. (previously genogroup NT29) (Margos et al., 2013; Mukhacheva and Kovalev, 2013). Since it is known that the clinical outcome of borreliosis may depend on the pathogen species (Balmelli and Piffaretti, 1995), great importance is paid to the differential diagnosis of *Borrelia* species in clinical material as well as in ticks. There are several techniques of *Borrelia* differentiation based on conventional PCR, real-time PCR and reverse line blot, as well as low-density microarrays. However, most of these techniques were developed by American researchers to differentiate genospecies prevalent in North America: *B. burgdorferi* s.s., *B. bissettii*, *B. miyamotoi*, and *B. lonestari* (Barbour et al., 2009; Houck et al., 2011; Tsao et al., 2004; Ullmann et al., 2005). Some of the techniques, including the classical method PCR-RFLP of the rrf-rrl intergenic spacer (Postic et al., 1994), are focused exclusively on European genospecies of *B. burgdorferi* s.l. (Demaerschalck et al., 1995; Portnoi et al., 2006; Rijpkema et al., 1995). Since the *B. garinii* genogroup NT29 was delineated into a new species, *B. bavariensis*, some currently used techniques have been adapted to differentiate these species (Herrmann et al., 2013; Scholz et al., 2013). However, no method based on the TaqMan approach has been proposed for this purpose despite its specificity, the possibility of multiplexing and the quantitative analysis of each target.

Some techniques based on both conventional and real-time PCR were developed to differentiate *B. miyamotoi* and *B. burgdorferi* s.l. To increase the reliability of these techniques, group-specific genes are used (Borgoyakov et al., 2011; Portnoi et al., 2006). For example,

* Corresponding author. Tel.: +7 343 2616826; fax: +7 343 3507401.
E-mail address: Sergey.Kovalev@urfu.ru (S.Y. Kovalev).

http://dx.doi.org/10.1016/j.ttbdis.2014.05.016
1877-959X/

gene glpQ (glycerophosphodiester phosphodiesterase) has been shown to be specific for TBRF *Borrelia* such as *B. miyamotoi* (Schwan et al., 1996).

In addition to studying *Borrelia* species composition in ticks or clinical material, a technique of rapid simultaneous genotyping of *Borrelia* should be used as a preliminary step before carrying out multilocus sequence typing (Margos et al., 2008). Such a genotyping technique could allow identification of individual *Borrelia* species for further sequencing. As the MLST procedure requires unambiguous sequences for eight genes, the presence of two genospecies may lead to unreliable interpretation of data. Such complication has been reported repeatedly when carrying out MLST analysis because of the high incidence of mixed *Borrelia* infections in ticks (Mukhacheva and Kovalev, 2013; Scholz et al., 2013; Vollmer et al., 2011). A preliminary stage of species differentiation could help to avoid ambiguous sequences, which should be excluded from further analysis. In addition, it facilitates the selection of species of particular interest, as well as samples with a DNA concentration suitable for subsequent typing by MLST.

The aim of this study was to estimate the prevalence of clinically important *Borrelia* species in Russia by newly designed primers and probes, as well as to evaluate the possibility of their use as a preliminary step for MLST.

Materials and methods

Individual adult questing ticks, *I. persulcatus* (1343 specimens, collected by flagging from North-West Russia to the Far East between 2009 and 2013 years) and *I. pavlovskyi* (92 specimens from the Tomsk region), were subjected to analysis (Table 2, Fig. 2). Ticks were frozen in liquid nitrogen and homogenized in saline. Nucleic acids were extracted using commercial kits Ribo-prep or MAG-NOsorb (InterLabService, Moscow). Reverse transcription reaction was performed using Reverta-L (InterLabService). cDNA was used to detect *Borrelia* sp. (target gene – 16 S rRNA) according to the procedure of Ornstein and Barbour (2006), with minor modifications. Real-time PCR was performed on an ABI 7500 thermocycler (Applied Biosystems, USA). The PCR conditions were 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 s, 60 °C (primer sets №1 and №3) or 58 °C (primer set №2) for 20 s, and 60 °C for 40 s. Differentiation of four genospecies in positive samples was carried out in two stages (Fig. 1). In the first stage, we detected separately *B. afzelii, B. garinii* s.l. (*B. garinii* + *B. bavariensis*) and *B. miyamotoi* using the primer set №1 (Table 1, Fig. 1). In the second stage, *B. garinii* s.l.-positive samples were differentiated into *B. bavariensis* and *B. garinii* using primer set №2. Sequences of primers and probes used are given in Table 1. Primers and probes were designed using Vector NTI 10.3.0 (Invitrogen) and Primer Express 3.0 (Applied Biosystems) based on the sequence alignment of selected genes, uvrA (excinuclease ABC subunit A) and nifS (aminotransferase) for *B. burgdorferi* s.l. and glpQ for *B. miyamotoi*, obtained from GenBank and the Borrelia MLST database (http://borrelia.mlst.net/). Specificity of the primers and probes was tested on strains of *B. afzelii*, *B. bavariensis* and *B. garinii* typed by MLST (Margos et al., 2008), as well as isolates of *B. miyamotoi*. For selected non-typed samples, fragments of the uvrA and nifS genes that contain binding sites for the primers and probes were amplified by conventional nested PCR and sequenced. For ticks from the Tomsk and Novosibirsk regions, as well as those from Primorsky Krai (regions where *I. persulcatus* and *I. pavlovskyi* are sympatric), tick species identification was carried out by real-time PCR (primer set №3) (Table 1).

Results

The prevalence of *Borrelia* sp. ranged from 21 to 57% among the localities studied (on average 40%) (Table 2). The highest

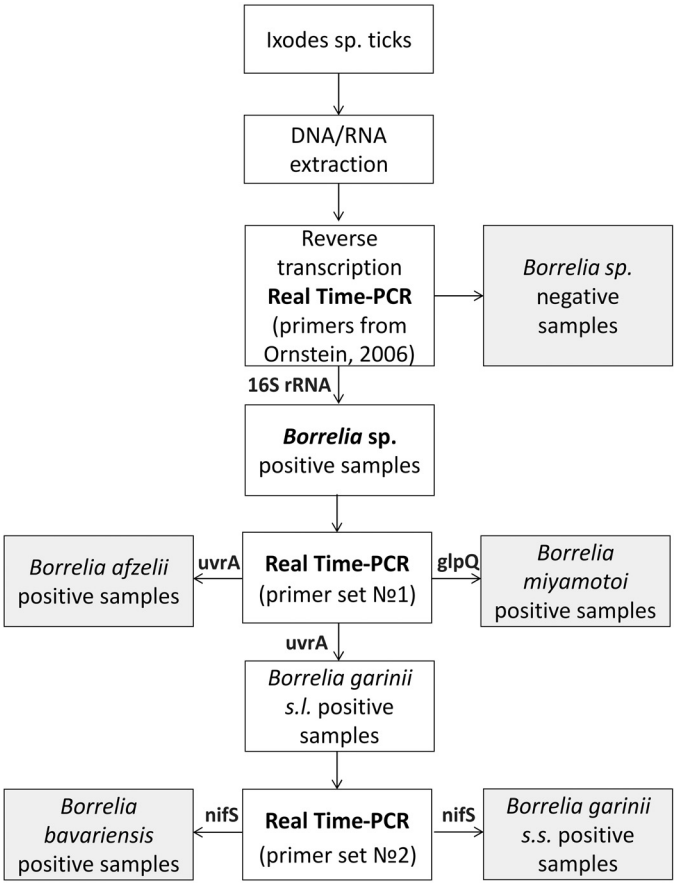

Fig. 1. The scheme of consecutive differentiation of *Borrelia* sp. genospecies. The RT-PCR targeted genes are indicated above the arrows.

prevalence was reported in the Perm region (57%), the lowest in the Tyumen region (21%). No clear pattern of genospecies distribution was revealed. *I. persulcatus* and *I. pavlovskyi* ticks were infected by *Borrelia* sp. at almost the same rate (41 and 45%, respectively). Unfortunately, in some cases we were not able to determine any species though the sample was *Borrelia*-positive (on average, 10% of positive samples were non-typed). Selective sequencing of gene fragments containing binding sites for the primers and probes showed no nucleotide substitution, except in one case in which one nucleotide substitution was found in the primer B_uvrA_R and a degenerate nucleotide was therefore included in the primer sequence. Although the presence of non-typed samples may be due to genetic polymorphism and, consequently, incomplete specificity of the primers and probes used, the most likely reason is a lower sensitivity of primers for differentiation than for detection purposes.

The average prevalence of *B. miyamotoi* in *I. persulcatus* ticks was the lowest, i.e. about 10% from the number of *Borrelia*-positive samples or 4% from the number of ticks studied (Table 2), which corresponds to the data shown previously (Fomenko et al., 2010; Platonov et al., 2011). *B. afzelii* was the most prevalent genospecies in *I. persulcatus* (27 to 65%, depending on locality). Particular attention must be paid to the peculiarities of the spatial distribution of *B. bavariensis* and *B. garinii*, previously considered as a single species (Fig. 2). Mixed infections of these two species were extremely rare (on average 2%) (Table 2). In the European part of Russia (Fig. 2: places 1 and 2), *B. garinii* was virtually absent in *I. persulcatus* ticks, whereas in the Asian part proportions of *B. bavariensis* and *B. garinii* are almost equal (Fig. 2: places 3,5,6 and 7) with constant, although not significant, domination of *B. bavariensis*. Surprisingly,