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Original article

A newly established real-time PCR for detection of *Borrelia miyamotoi* in *Ixodes ricinus* ticks

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ABSTRACT

A total of 350 ticks collected in Austria were analyzed for the presence of DNA sequences of *B. miyamotoi*. Three ticks gave positive results in a *B. miyamotoi*-specific nested PCR. Results were confirmed by sequencing the amplified *glpQ* gene from the positive samples. Moreover we developed a real-time PCR which unambiguously detected *B. miyamotoi* in all positive samples. Further genotyping of the samples found 100% identity of the 16S–23S intergenic spacer region with Swedish *B. miyamotoi* sequences. This is the first detection of the relapsing fever spirochete *Borrelia miyamotoi* in hard ticks in Austria. The results consolidate the picture of a European-wide distribution of *B. miyamotoi* and again underscore the need for clinical awareness to clarify possible involvement of this species in human disease.

Introduction

Spirochetes of the genus *Borrelia* are currently subdivided into two major groups (Geller et al., 2012): the relapsing fever (RF) borreliae, which are harbored and transmitted by the body louse *Pediculus corporis* (louse borne-RF) or by argasid or soft ticks (tick-borne RF), and the spirochetes of the *Borrelia burgdorferi* sensu lato (sl) complex, which are transmitted by ixodid or hard ticks. The *B. burgdorferi* sl complex includes the causative agents of Lyme borreliosis (LB) (Barbour et al., 2009), which presents predominantly with manifestations of infection of the skin, nervous system, musculoskeletal system, rarely the heart and other organs, as well as with rare chronic courses of skin, joint and central nervous system involvement (Stanek et al., 2012). Tick-borne RF is mainly characterized by repeated episodes of high fever (Assous and Wilamowski, 2009).

There is a third group of borrelial species that are closely related to RF spirochetes but use ixodid ticks as vectors (Barbour et al., 2009). Representatives of this group include *B. theileri*, *B. lonestari* and *B. miyamotoi* (Barbour et al., 2009). Originally isolated from *Ixodes persulcatus* on the island of Hokkaido, Japan, in 1995 (Fukunaga et al., 1995), *B. miyamotoi* has been found in hard ticks of different species, such as *I. scapularis*, *I. ricinus* and *I. pacificus* (Platonov et al., 2011). The detection of *B. miyamotoi* in Japan (Fukunaga et al., 1995), in USA (Scoles et al., 2001; Mun et al., 2006) and in Eurasia (Fraenkel et al., 2002; Richter et al., 2003; Fomenko et al., 2010; Hansford et al., 2014) indicates the wide distribution of this species in the northern hemisphere. Analysis of the flagellin gene and 16S rRNA has revealed a closer relationship to other RF borreliae than to Lyme borreliae (Fukunaga and Koreki, 1995; Fukunaga et al., 1995). At the genomic level, RF and Lyme borreliae are easily distinguished by the presence (Lyme borreliae) or absence (RF borreliae) of tandemly repeated 5S and 23S ribosomal genes (Davidson et al., 1992; Schwartz et al., 1992). The highly variable region of the 5S-23S duplication has been extensively used for subtyping *B. burgdorferi* sl species (Wang et al., 1999). *B. miyamotoi* lacks this second copy of the ribosomal genes, again demonstrating the closer relationship to other RF borreliae (Fukunaga et al., 1995).

Another gene (*glpQ*) encoding a glycerophosphodiester phosphodiesterase is present only in RF borreliae but not in Lyme borreliae (Schwan et al., 1996; Fraser et al., 1997); this gene has been used for detection of RF borreliae and for specific identification and characterization of *B. miyamotoi* (Fomenko et al., 2010; Geller et al., 2012; Hansford et al., 2014). A third gene, the intergenic spacer (IGS) between the 16S and 23S genes is present in both Lyme borreliae and RF borreliae and is commonly used for characterization of both groups (Liveris et al., 1995; Bunikis et al., 2004b). Although the IGS exhibits greater diversity in

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B. miyamotoi than in other borreliae it remains a useful locus for genotyping RF borreliae (Bunikis et al., 2004b).

The clinical importance of *B. miyamotoi* is currently under discussion. Although humans might become infected with *B. miyamotoi*, evidence for causing human disease is currently weak (Hamer et al., 2012; Fonville et al., 2014). Nevertheless, the number of scientific papers and case reports demonstrating association of *B. miyamotoi* with RF-like and LB-like symptoms in patients is increasing and *B. miyamotoi* has been identified in ticks in several European countries and in patients (Richter et al., 2003; Platonov et al., 2011; Geller et al., 2012; Hovius et al., 2013; Krause et al., 2013). The aim of the present study was to identify *B. miyamotoi* in ticks collected in Austria and to develop a rapid and reliable detection method for this species.

Materials and methods

Ticks

Ticks were collected by flagging during spring/summer 2005 in all the federal states of Austria; ticks from Vienna were collected separately in autumn 2013. The 2005 Vienna batch was used up in another study. All ticks were frozen at −20 °C until processing. Altogether, 350 *Ixodes ricinus* ticks comprising 46 adults (22 females, 24 males), 302 nymphs and 2 larvae were used for the study.

DNA extraction from ticks

DNA was extracted from the ticks by alkaline lysis (Guy and Stanek, 1991; Rijpkema et al., 1996) or with the DNeasy Blood and Tissue kit (Qiagen, Germany), using a modified protocol. Briefly, ticks were lysed overnight in 180 μl ATL buffer and 20 μl proteinase K solution at 56 °C while shaking. AL buffer (200 μl) was then added, followed by incubation at 70 °C for 10 min. After the addition of 200 μl 96% ethanol the mixture was centrifuged at 18,000 × *g* for 5 min. The remaining steps were as described in the manufacturer's protocol. A negative extraction control was included with each batch in order to exclude possible cross-contamination during the extraction process.

PCR and real-time PCR

For the detection of *B. miyamotoi*, DNA samples extracted from groups of up to 10 ticks were pooled for testing; the ticks in any *B. miyamotoi*-positive pools were then tested individually. A nested PCR was used as described previously (Fomenko et al., 2010). The master mix contained (final concentrations): 0.4 μM each primer, 400 μM dNTPs (Solis BioDyne, Estonia), 1.25 u peqGOLD Hot Taq-DNA-Polymerase (PeqLab, Germany), 5 μl reaction buffer Y (PeqLab, Germany), 10 μl enhancer solution P (PeqLab, Germany), 5 μl extracted DNA. Reaction volumes were adjusted to 50 μl with PCR-grade water (Sigma–Aldrich, Austria). Primers Q1/Q2 were used for the first round of amplification. The reaction conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 45 s, and a final elongation step of 72 °C for 7 min. Primers Q3/Q4 were used for the second round of amplification and reaction conditions for the nested amplification were the same as for the first round, except that the annealing temperature was 52 °C. A negative control containing PCR-grade water in the PCR master-mix instead of DNA was included in every run to control for possible contamination.

The *Borrelia rrs–rrlA* region was amplified in a nested PCR, as described elsewhere (Bunikis et al., 2004a). The master mix was prepared as above. Primers rrs–rrlA IGS F/rrs–rrlA IGS R were used in the first round of amplification. Reaction conditions were 95 °C for 7 min, followed by 35 cycles at 95 °C for 45 s, 55.5 °C for 1 min and 72 °C for 45 s, and a final elongation step of 72 °C for 7 min. Primers rrs–rrlA IGS Fn/rrs–rrlA IGS Rn were used for the second round of amplification. Reaction conditions for nested amplification were the same as for the first round.

A PCR with primers S1/S2 targeting *B. miyamotoi* 16S rDNA was run with the following reaction conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 90 s; the final extension step was at 72 °C for 7 min. Portions (5 μl) of the reaction product were used as template to generate PCR fragments suitable for cloning. For cloning of *B. miyamotoi* 16S rDNA, primer pairs Bm16S_Fwd_A/Bm16S_Rev_A and Bm16S_Fwd_B/Bm16S_Rev_B were used to generate two fragments 620 bp and 762 bp in length, respectively. Reaction conditions were as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 52 °C for 45 s and 72 °C for 1 min, and a final elongation step at 72 °C for 7 min.

For real-time PCR in an Applied Biosystems 7500 Real-Time PCR Cycler, primers and probes were based on sequences available from the National Center for Biotechnology Information (NCBI) (accession numbers GI:253757016, GI:38230732, GI:237866044). A complete match with *B. miyamotoi* strain LB-2001 (GI:60396860) could not be achieved. The reactions contained 1 μl primer/probe mix (ingenetix, Austria), 10 μl 2× TaqMan Gene Expression Master Mix (Life Technologies, Austria) and 4 μl of tick DNA extract. The reaction volume was adjusted to 20 μl with PCR-grade water.

The following conditions were used in the 2-step PCR: 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 56 °C for 2 min. For specificity testing, crude DNA extracts of the following strains were used: *B. turicatae*, *B. duttonii*, *B. hermsii* and *B. burgdorferi* B31. For sensitivity testing, vector pBBmglpQ was purified from *E. coli* DH5-α using a plasmid mini kit (Fisher Scientific, Vienna, Austria) and quantity was determined photometrically. Copy number was calculated from the molecular weight of pBBmglpQ. The extract was then serially diluted and the highest dilution resulted in 0.1 copy of the plasmid within the PCR reaction (Fig. 2). We additionally tested the sensitivity of the assay, when tick-DNA lysates were used instead of water for serial dilution of vector pBBmglpQ. Therefore, the genomic DNA of 15 nymphal *I. ricinus* ticks was extracted using the DNeasy Blood and Tissue kit (Qiagen, Germany) as described above. Subsequently all extracts were tested with the real-time PCR assay to ensure they were *B. miyamotoi* negative. After that all extracts were pooled together and used for the dilution series of the experiment (Fig. 2).

Sequences of all primers and probes used in this study are listed in Table 2.

Cloning strategies

A plasmid mini kit was used to purify the pBluescript KS II+ vector from an overnight culture of *E. coli* DH5-α.

TA cloning of glpQ

The *glpQ* gene fragment was amplified from one of the tick extractions as described above and purified using a commercially available kit (Qiagen, Germany). The vector was digested with EcoRV (Fisher Scientific, Austria) according to the manufacturer's instructions. The digested and purified vector was then incubated for 2 h at 72 °C with Taq polymerase (Fisher Scientific, Austria) in the presence of 2 mM dTTP (Solis BioDyne, Estonia). The *glpQ* fragment was ligated into the resulting T-vector using T4 Ligase (Fisher Scientific, Austria), resulting in vector pBBmglpQ. Subsequently, *E. coli* DH5-α cells were transformed and clones were verified by sequencing (MWG Eurofins, Germany).