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Rapid Differentiation of *Borrelia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi* Sensu Stricto by LightCycler Fluorescence Melting Curve Analysis of a PCR Product of the *recA* Gene

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To differentiate the *Borrelia burgdorferi* sensu lato genospecies, LightCycler real-time PCR was used for the fluorescence (SYBR Green I) melting curve analysis of borrelial *recA* gene PCR products. The specific melting temperature analyzed is a function of the GC/AT ratio, length, and nucleotide sequence of the amplified product. A total of 32 DNA samples were tested. Of them three were isolated from *B. burgdorferi* reference strains and 16 were isolated from *B. burgdorferi* strains cultured from *Ixodes ricinus* ticks; 13 were directly isolated from nine human biopsy specimens and four *I. ricinus* tick midguts. The melting temperature of *B. garinii* was 2°C lower than that of *B. burgdorferi* sensu stricto and *B. afzelii*. Melting curve analysis offers a rapid alternative for identification and detection of *B. burgdorferi* sensu lato genospecies.

Borrelia burgdorferi sensu stricto, *B. garinii*, and *B. afzelii* are the genospecies of *B. burgdorferi* sensu lato proven to be responsible for human Lyme borreliosis (19). In Europe, *B. garinii* and *B. afzelii* are the most prevalent genospecies, whereas *B. burgdorferi* sensu stricto is the only genospecies encountered in North America (19). For the genotypic identification of *B. burgdorferi* sensu lato genospecies, PCR and PCR-based assays, such as species-specific PCR (8, 10), randomly amplified polymorphic DNA analysis (20, 21), PCR-based sequencing (4–6, 11, 18), and restriction fragment length polymorphism (9, 13, 14), are commonly used. All of these methods, although they have the advantage of high discriminative power, are considered to be relatively laborious and expensive. Recently, Morrison et al. (12) described a continuous fluorescence-monitoring PCR for the *recA* gene of *B. burgdorferi* sensu stricto. The *recA* gene belongs to a set of genes responsible for homologous recombination in bacteria (3). The gene is located on the chromosome of the spirochete and thought to be evolutionally conserved (3, 12). Using primers described by Morrison et al. (12), we analyzed the melting curves of PCR products derived from the different genospecies of *B. burgdorferi* sensu lato.

A total of 32 DNA samples were tested. Of them 3 were isolated from *B. burgdorferi* reference strains and 16 were isolated from *B. burgdorferi* strains cultured from *Ixodes ricinus* tick midguts; 13 were directly isolated from nine human biopsy specimens punched from marginal areas of erythema migrans lesions and four *I. ricinus* ticks from which the midgut was removed (Table 1). The human biopsy specimens obtained were first cut in half by scissors. One half was cultured and from the other half DNA was extracted directly and used for PCR. The midgut of each tick was removed by tweezers under a microscope and cultured. DNA was extracted directly from the ticks from which the midgut was removed. The specimens submitted to culture were inoculated into tubes containing Barbour-Stoenner-Kelly (BSK-II) medium (1) and incubated at 30°C for 4 to 6 weeks. The tubes were observed macroscopically twice a week. Dark-field microscopy was carried out if the color indicated growth. The final identification of cultured spirochetes was based on PCR and sequencing (7, 17). The DNA was extracted from cultured bacteria and directly from human and tick tissues using InstaGene matrix (Bio-Rad, Hercules, Calif.), according to the manufacturer's instructions.

PCR was performed using a fluorescence temperature cycler (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany). The primers were the same as those used by Morrison et al. (12) and flanked a product 222 bp in length. The primers were nTM17.F (5′ GTG GAT CTA TTG TAT TAG ATG AGG CTC TCG 3′) and nTM17.R (5′GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG 3′). Amplification was done according to the general guidelines provided by the manufacturer of the LightCycler after optimization of the various reaction parameters. The 20-μl reaction volume in a glass capillary contained 2 μl of LightCycler-DNA Master SYBR Green I mix (*Taq* DNA polymerase, reaction buffer, deoxynucleoside triphosphate mix, and SYBR Green I dye), 3 mM $MgCl_2$, 20 ng of bovine serum albumin [BSA], an 8 μM concentration of each primer, 220 ng of TaqStart antibody (ClonTech, Palo Alto, Calif.), and 4 μl of extracted DNA. DNA preparations (concentration, ~2 μg/ml) extracted from the reference strains of *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* were used as positive controls, and mixtures of all reagents, devoid of added DNA, were used as negative controls. The three positive controls and one negative control were included in each PCR run.

The amplification program included the initial denaturation step at 95°C for 40 s and 50 cycles of denaturation at 95°C for 1 s, annealing at 59°C for 5 s, and extension at 72°C for 11 s. The temperature transition rate was 20°C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 55°C, keeping it at 55°C for 20 s, and then slowly heating it at 0.1°C/s to 94°C. Fluorescence was measured through the slow heating phase. For improved visualization of the melting temperatures (T_m), melting peaks were derived from the initial melting curves (fluorescence [F] versus temperature [T]) by plotting the negative

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TABLE 1. Origins and T_m analysis of strains used in this study[a]

Species and sample (*n*)	Location (yr) of isolation	Mean T_m ± SD (°C)
B. burgdorferi sensu stricto ATCC 35210 (1)	United States	84.02
B. garinii		
387 CSF (1)	Germany	81.62
Human samples (8)	Finland (1996–1999)	80.62 ± 0.33[b]
Tick samples (10)	Finland (1996–1998)	81.30 ± 0.40
B. afzelii		
Bo 23 (1)	Germany	83.71
Human samples (1)	Finland (1999)	83.84
Tick samples (10)	Finland (1998–1999)	83.42 ± 0.27

[a] DNA was extracted from the *Borrelia* reference strain, human skin biopsy samples, and tick midguts and was subjected to real-time PCR for the *recA* gene; melting curve analysis was also performed.

[b] $P = 0.0014$ when compared to T_m of *recA* PCR products amplified from tick samples.

derivative of fluorescence over temperature versus temperature ($-dF/dT$ versus T). Melting curves were used to determine the specific PCR products (15), which were further confirmed using conventional gel electrophoresis.

The Student t test was used to analyze statistical significance. All P values corresponded to two-tailed tests, and a P of <0.05 was considered statistically significant.

The mean T_m values for the reference strains of *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, obtained by testing aliquots of the same sample in five reaction capillaries during the same run, were 84.02, 83.71, and 81.62°C, respectively (Table 1 and Fig. 1). The corresponding intra-assay coefficients of variation were 0.08, 0.05, and 0.06%. The mean T_m values for the reference strains of *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, obtained by testing aliquots of the same sample during six separate runs, were 83.57, 83.54, and 81.42°C, respectively (Table 1). The corresponding interassay coefficients of variation were 0.4, 0.4, and 0.5%. To test the effect of target DNA concentration on T_m, six fourfold dilutions of *B. garinii* DNA (starting concentration, ~2 μg/ml)

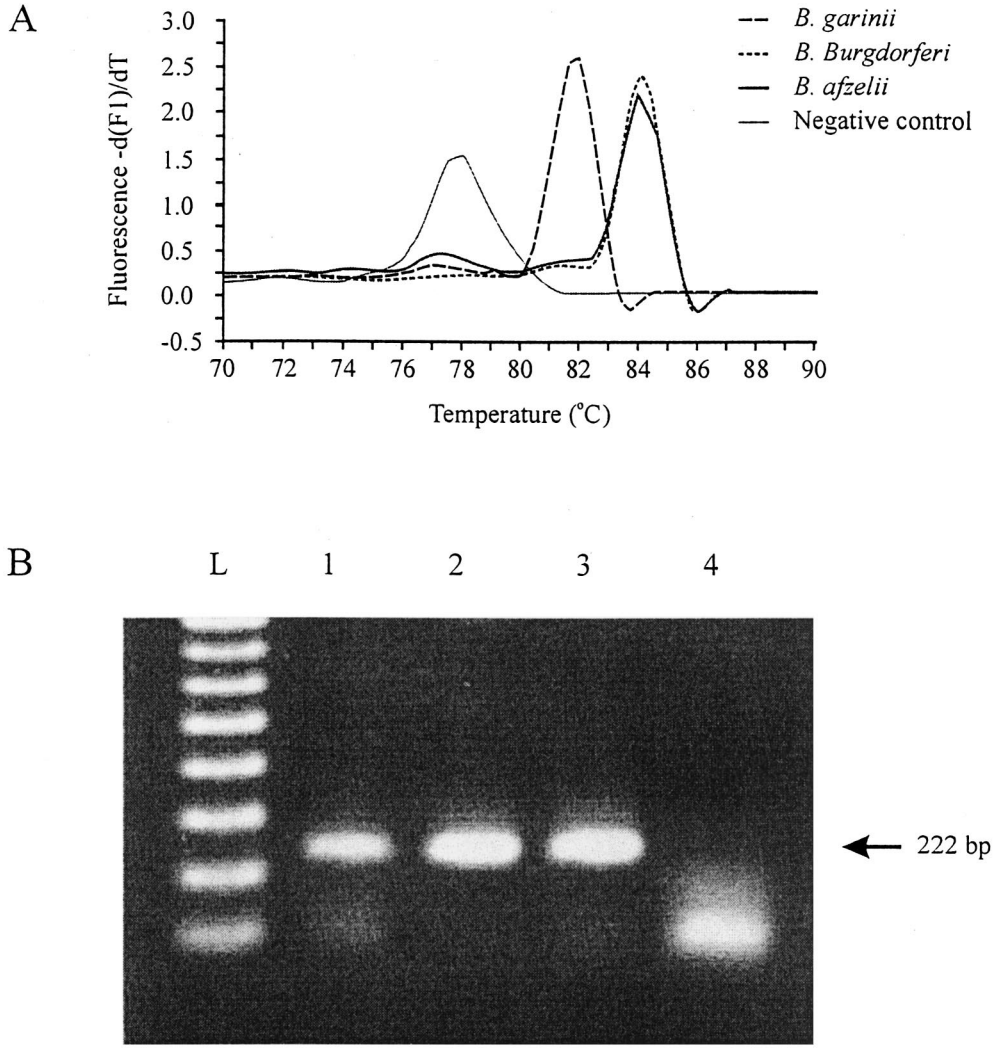

FIG. 1. Using the real-time PCR, a fragment of the *recA* gene was amplified from the genospecies of *B. burgdorferi* sensu lato, and fluorescence melting curve analyses as well as gel electrophoresis of the products were done. (A) The T_m was 81.62°C for *B. garinii*, 84.02°C for *B. burgdorferi* sensu stricto, and 83.71°C for *B. afzelii*. Primer-dimers in the negative control melted at temperatures below 80°C. (B) Lane L contains molecular weight markers (100 bp), and lanes 1 to 4 show PCR products obtained from *B. garinii*, *B. burgdorferi* sensu stricto, *B. afzelii*, and the negative control, respectively.

were tested during the same run, and the mean T_m was 81.77°C (standard deviation [SD], 0.41).

The mean T_m values obtained from the DNA derived from the tick samples harboring *B. garinii* ($n = 10$) or *B. afzelii* ($n = 10$) were 81.30°C (SD, 0.40) and 83.42°C (SD, 0.27), respectively (Table 1). The mean T_m obtained from human samples ($n = 8$) harboring *B. garinii* was 80.62°C (SD, 0.33). A statistically significant difference was found between the T_m of *B. garinii* DNA isolated directly from human clinical specimens and the T_m of borrelial DNA of tick origin ($P = 0.0014$). The T_m of one human specimen positive for *B. afzelii* was 83.84°C.

To our knowledge, this is the first report on the use of the fluorescence melting curve analysis of PCR products to identify the genospecies of *B. burgdorferi* sensu lato. Compared with conventional PCR and PCR-based assays, this analysis system is less complex and decisively more rapid, with results obtained within 1 hour. Our preliminary results show that using the present real-time PCR we can reach the same sensitivity (about five organisms of *B. burgdorferi* including all three genospecies of *B. burgdorferi* sensu lato) as using the nested PCR used in our laboratory (7, 17). This suggests that the LightCycler PCR can be used for the detection and differentiation of *B. burgdorferi* directly in clinical samples.

It is known that the DNA extracted from clinical samples, such as blood and tissue, contains inhibitors. These inhibitors can interfere with the PCR-based detection of microbial infection and cause false-negative results. It has been reported that pretreatment of DNA with or addition of BSA into the PCR mixture can remove inhibitors (2, 16). This is why in this study BSA was added to all reaction mixtures. Further, a relatively small volume of DNA was used for the PCR to minimize the inhibitory action of the sample.

The intra- and interassay variation coefficients of the T_m values were very low, showing that the technical principles of the LightCycler allow consistent temperature conditions for the reaction capillaries. The difference between the T_m values of the reference strains *B. garinii* and *B. afzelii* or *B. burgdorferi* sensu stricto was about 2°C. A similar difference between *B. garinii* and the other genospecies was found when DNA isolated from clinical samples was tested. The shape and position of the DNA melting curve are functions of the GC/AT ratio, length, and sequence (15). Ririe et al. (15) used the LightCycler to analyze the melting curves of a mixture of a 180-bp fragment of the hepatitis B surface antigen gene and a 536-bp fragment of the human β-globin gene. They concluded that PCR products with T_m differences of even less than 2°C can be distinguished (15).

The T_m values of *B. garinii* DNA isolated directly from human clinical specimens and borrelial DNA of tick origin showed differences which were statistically significant. This may reflect the change that the bacterium has to undergo during its adaptation to different environments. However, the effect of the matrix from which the DNA was extracted cannot be totally excluded.

During PCR amplification, unspecific products, even primer-dimers that are produced when little or no template is present, can be formed. Because these products are double stranded, they also bind SYBR Green I dye and give a signal. The analysis of melting curves can differentiate the unspecific products from the specific product, because the T_m of unspecific products will be lower. In our PCR, all unspecific products melted at temperatures below 80°C.

The difference in T_m between *B. burgdorferi* sensu stricto and *B. afzelii* reference strains was so small (84.02 versus 83.71°C) that it cannot be used for distinguishing these two species. However, these two species could possibly be differentiated if a gene with a wider heterogeneity is used for the LightCycler PCR and melting curve analysis. Rapid differentiation of *B. garinii* and *B. afzelii* is highly valuable in Europe, where these genospecies are prevalent and *B. burgdorferi* sensu stricto is rarely encountered.

Our results indicate that the fluorescence melting curve analysis of *recA* PCR products can be used for differentiation of *B. garinii* from *B. afzelii* and *B. burgdorferi* sensu stricto genospecies. Studies are under way to sequence the amplified fragments for determination of factors underlying T_m differences. Further, the fluorescence melting curve analysis of PCR products could be applied to differentiation between other bacterial species, provided that gene segments with suitable variation are available.

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