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Sensitive Detection of *Borrelia burgdorferi* Sensu Lato DNA and Differentiation of *Borrelia* Species by LightCycler PCR

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In order to differentiate species within the *Borrelia burgdorferi* sensu lato complex, LightCyler PCR and melting-curve analysis of the amplicons of two genes with intraspecies variability, the *p66* gene and the *recA* gene, were performed. It was demonstrated that nested LightCycler PCR amplification of *p66* is more sensitive in the detection of borrelia DNA than amplification of the *recA* gene. *B. burgdorferi* sensu stricto could be differentiated from *Borrelia garinii* and *Borrelia afzelii* by melting-curve analysis of the *p66* gene amplicon. *B. garinii* could be differentiated from *B. afzelii* and *B. burgdorferi* sensu stricto by melting-curve analysis of the *recA* gene amplicon. Therefore, the PCRs complement each other in subtyping different *Borrelia* species, and combined LightCycler PCR and melting-curve analysis of both target genes is a rapid method to distinguish the three species of *B. burgdorferi* sensu lato.

Lyme disease is the most prevalent tick-borne disease of the Northern Hemisphere (3). Its etiologic agent, *Borrelia burgdorferi* sensu lato, has been divided into different species. *B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii* are the common human pathogenic species (4, 21). The infection leads to a variety of clinical symptoms involving the skin, nervous system, heart, and joints (19).

Erythema migrans (EM) and acrodermatitis chronica atrophicans (ACA) represent common cutaneous manifestations of an infection by *B. burgdorferi* sensu lato (1, 13, 21), whereas the role of this spirochete in the pathogenesis of morphea and lichen sclerosus et atrophicus is controversial (13, 15, 23, 25).

Since PCR has proved to be a sensitive and fast method for the diagnosis of microrganisms which are difficult to culture, the technique has been applied to the detection of *B. burgdorferi* sensu lato DNA in infected ticks (6) as well as in human specimens, such as cerebrospinal fluid (4) or synovial fluid (5, 20), urine (2, 16), and skin (9, 24). Established PCR protocols amplify different segments of borrelial chromosomal genes, such as the flagellin gene (10, 15), the one-copy 16S rRNA gene (7), the 23S rRNA gene (17), the *p66* gene segment encoding a 66-kDa protein (14), the *recA* gene (8), and the plasmid-encoded *ospA* gene (9, 20).

Common PCR with a conventional thermocycler and subsequent separation of the amplicon by agarose gel electrophoresis allows the detection of *B. burgdorferi* sensu lato DNA. Subtyping of *Borrelia* species DNA is not possible, since the intraspecies sequence polymorphisms of PCR amplicons are only a few base pairs long.

Several postamplification methods were employed to identify *Borrelia* species commonly associated with Lyme borreliosis, e.g., oligonucleotide typing with PCR fragments (5), randomly amplified polymorphic DNA fingerprinting analysis (22), pulsed-field gel electrophoresis (4), single-strand conformation polymorphism (18), and subtype-specific PCR targeting the 16S rRNA gene (6). These techniques are usually time-consuming, and some of them require high technical standards and experience.

LightCycler PCR with melting-curve analysis is a new, rapid method to perform PCR and to analyze sequence variations of the amplified fragments without the need of additional techniques by performing a melting-temperature (T_m) analysis immediately after amplification is completed. The specific T_m of a DNA template is defined as the temperature at which 50% of the duplices become single stranded. It is influenced by the GC content, length, and nucleotide sequence of the amplified product (27, 28).

A recent report showed that the amplification of the *recA* gene with a single primer set leads to the differentiation of *B. garinii* DNA from *B. afzelii* and *B. burgdorferi* sensu stricto DNA by its lower specific T_m. However, the difference in T_m between *B. afzelii* and *B. burgdorferi* sensu stricto was too small to distinguish the two species (11). To improve species differentiation, we evaluated a PCR of another target gene with intraspecies variability on the LightCycler system and analyzed the melting curves derived from three species within the *B. burgdorferi* sensu lato complex, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, and compared these melting profiles with the results obtained by analyzing *Borrelia recA* gene PCR products of the same samples.

For this purpose, we chose a nested PCR targeting the *p66* gene segment, which was originally described by Rosa and Schwan (14), modified by Wienecke et al. (24), and which proved to be highly specific and sensitive (12, 25). The 92-bp amplified target sequence of this gene segment differs at various positions among the three *Borrelia* species, as indicated in Fig. 1. Sequences were obtained from GenBank; the accession numbers are as follows: *B. burgdorferi* target sequence, M58431.1; *B. garinii*, X87727.1; *B. burgdorferi* sensu stricto, X87725.1; and *B. afzelii*, X87726.1. Potential T_ms for these amplicons were calculated by the oligoapplet program available from TIB MOLBIOL, Berlin, Germany, which revealed

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FIG. 1. Sequence comparison of the 92-bp amplicon of the *p66* genes of *B. garinii, B. afzelii*, and *B. burgdorferi* sensu stricto (as obtained from GenBank) with the results of patients' samples (as obtained by sequencing the amplified PCR products). Variant base positions are indicated by underlining. The primer positions are shown below.

T_ms of 79.0°C for *B. afzelii*, 79.0°C for *B. garinii*, and 81.7°C for *B. burgdorferi* sensu stricto (Table 1).

We analyzed DNA of *Borrelia* control strains and eight patient samples. The DNA of the species *B. burgdorferi* sensu stricto (strain B31), *B. afzelii* (strain NE 632) (kindly provided by W. Bautsch, Hannover Medical University, Hannover, Germany), *B. garinii*, and *Borrelia hermsii* (purchased from Deutsche Sammlung von Mikroorganismen and Zellkulturen, Braunschweig, Germany) was extracted with a QIA Amp DNA isolation kit (Qiagen, Hilden, Germany).

B. hermsii served as a negative control, since it is not amplified by the primers used in this study. The skin biopsy specimens were obtained from eight patients with clear diagnosis of cutaneous borreliosis. The diagnosis was based on clinical data, histological data, and serological detection of elevated *B. burgdorferi* immunglobulin M and immunoglobulin G antibodies. Fresh frozen biopsy specimens were cut into small pieces, and genomic DNA extraction was performed with the QIA Amp DNA isolation kit.

PCR was performed on a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). The primers nTM17F and nTM17R (8) were used to amplify a 222-bp product of the *recA* gene. The PCR conditions and the LightCycler amplification and melting-curve program were reproduced exactly as described previously (11). For amplification of a 170-bp segment of the *p66* gene, the outer primer pair (Bb1 and Bb2) was

TABLE 1. T_m of *p66* and *recA* gene fragments[a]

Species (strain) or patient no.	Diagnosis	T_m (°C) calculated	Mean T_m (± SD) (°C)	
			p66 gene amplicon	*recA* gene amplicon
B. burgdorferi sensu stricto (B31)		81.7	78.72 ± 0.28	84.67 ± 0.28
B. afzelii (NE 632)		79.0	77.27 ± 0.33	84.20 ± 0.40
B. garinii		79.0	77.24 ± 0.37	82.89 ± 0.49
1	EM		77.65	84.24
2	EM		77.37	84.26
3	EM		77.57	84.62
4	EM		77.63	84.26
5	ACA		77.95	NA[b]
6	EM		76.87	NA
7	EM		77.15	NA
8	EM		79.23	NA

[a] T_ms of the *p66* gene fragment were calculated by the oligoapplet program and obtained by LightCycler PCR analysis of the *p66* gene amplicon and the *recA* gene amplicon of the three reference strains of *B. burgdorferi* sensu lato and of patients with EM or ACA. For the three reference strains, mean T_ms ± SD for 10 independent experiments are given.

[b] NA, no amplification of *Borrelia* DNA could be detected.

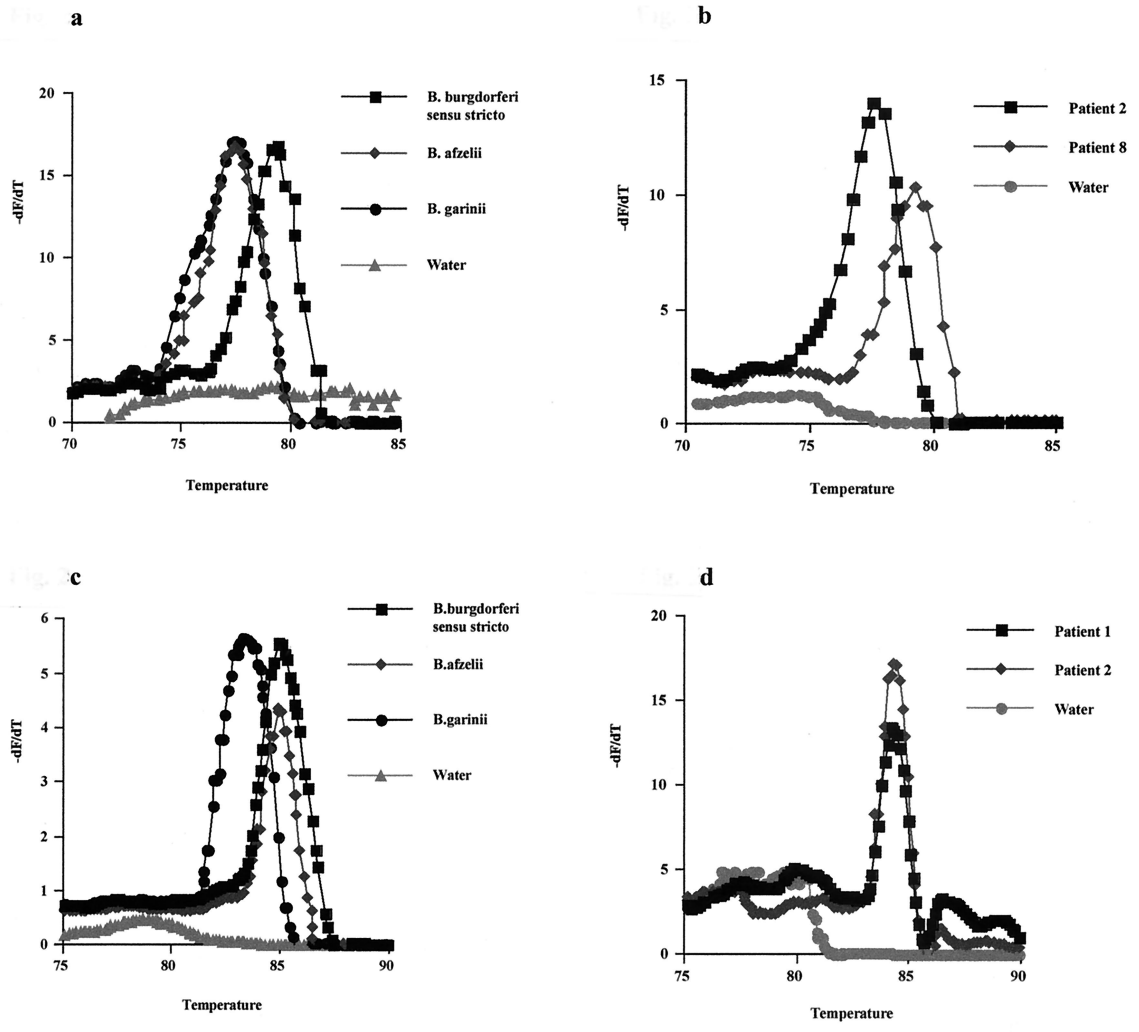

FIG. 2. Melting-curve analyses of amplification products of *p66* and *recA* genes from subspecies of *B. burgdorferi* sensu lato and two representative DNA samples of patients with skin manifestation of Lyme borreliosis. The T_m of the double-stranded fragment is visualized by plotting the negative derivative of the change of fluorescence (dF) divided by the change of temperature (dT) in relation to the absolute temperature. The turning point of this converted melting curve results in a peak and permits easy identification of the fragment-specific T_m. (a) Separation of *B. burgdorferi* sensu stricto from *B. afzelii* and *B. garinii* by melting-curve analysis of the *p66* gene amplicon. (b) Determination of *B. burgdorferi* sensu stricto in patient 8 and *B. afzelii* or *B. garinii* in patient 2 by melting-curve analysis of the *p66* gene amplicon. (c) Separation of *B. garinii* fom *B. burgdorferi* sensu stricto and *B. afzelii* by melting-curve analysis of the *recA* gene amplicon. (d) Determination of *B. burgdorferi* sensu stricto or *B. afzelii* in patients 1 and 2 by melting-curve analysis of the *recA* gene amplicon.

used. Subsequently, 2 μl of this PCR mixture was used as a template for a second run with the inner primer pair (Bb3 and Bb4) to amplify a 92-bp fragment (25) (Fig. 1).

Master mixes were based on a ready-to-use kit (Roche Diagnostics GmbH) containing *Taq* DNA polymerase, SYBR-Green I, and deoxynucleoside triphosphate mix (with UTP instead of TTP) and supplemented with 0.5 pmol of each primer and 3 mM $MgCl_2$.

Cycling was performed for both the outer and inner primer pairs for 40 cycles of denaturation (95°C for 1 s), annealing (55°C for 5 s), and extension (72°C for 12 s). After the final PCR cycle, the products were denatured at 95°C, annealed at 68°C, and then slowly heated to 95°C. During the slow heating process, fluorescence was measured continously at every 0.1°C. For analysis of the melting curves, the LightCycler instrument's software automatically converts them into melting peaks. The T_ms of the peaks were analyzed using the best-fit analysis software provided by Roche Molecular Biochemicals, and the mean T_ms are given for each sample in Table 1.

To assess the correct lengths of the fragments, 10 μl of the LightCycler PCR products was separated by agarose gel electrophoresis. As the DNA quality control, all skin samples were screened for human beta actin amplification with a primer set described by Wienecke et al. (25). To confirm *Borrelia* species identifications by their sequence-dependent T_ms, the *p66* gene products obtained by LightCycler PCR were purified using the Qia-quick PCR purification kit (Qiagen) and sequenced by BigDye terminator cycle sequencing (AB Applied Biosystems, Weiterstadt, Germany) on an automated PRISM 3700 capillary sequencer (AB Applied Biosystems).

T_ms of 77.27°C (standard deviation [SD], ±0.33) for *B. afzelii*, 77.24°C (SD, ±0.37) for *B. garinii*, and 78.72°C (SD, ±0.28) for *B. burgdorferi* sensu stricto were registered by LightCycler PCR and melting-curve analysis of the *p66* gene of *Borrelia* control strain DNA (Table 1 and Fig. 2a). In comparison with those of *B. afzelii* and *B. garinii*, the mean T_m of *B. burgdorferi* sensu stricto was shifted to 1.5°C higher due to the higher GC content of the amplified sequence. This was in accordance with the calculated T_ms showing a 2.7°C-higher T_m for *B. burgdorferi* sensu stricto than for *B. afzelii* and *B. garinii*. No amplification was observed with *B. hermsii* DNA as the target.

The LightCycler analysis was then applied to eight DNA samples from fresh frozen tissues of patients with serological, clinical, and histological diagnosis of cutaneous borreliosis. For six patients (patients 1, 2, 3, 4, 6, and 7) with a diagnosis of EM and one (patient 5) with a diagnosis of ACA, we found mean T_ms in a range of 77.15 to 77.95°C, similar to the values for the controls *B. afzelii* and *B. garinii*. One patient (patient 8) with a diagnosis of EM had a mean T_m of 79.23°C, which correlated with the melting profile of the control *B. burgdorferi* sensu stricto. T_m-defined groups were distinguished by a clear-cut separation of the melting curves (Fig. 2b). Unspecific products or primer dimers could be separated from specific products due to their T_ms, which were more than 5°C lower. A T_m around 72°C for the very small peak of the water control, caused by melting of unspecific primer dimers, was registered (Fig. 2b). Subsequent agarose gel electrophoresis of the LightCycler PCR products showed that bands of the appropriate size, 92 bp, were detected for positive results (data not shown). The 92-bp LightCycler PCR products of all patient samples were sequenced. All products which had mean T_ms between 77.15 and 77.95°C were identified as *B. afzelii*. The sample with a mean T_m of 79.23°C was identified as *B. burgdorferi* sensu stricto (Fig. 1).

Using nTM17F and nTM17R as primers, DNAs from the three *Borrelia* controls and from the eight patients were subjected to *recA* gene LightCycler PCR and melting-curve analysis. Mean T_ms for the controls *B. burgdorferi* sensu stricto B32, *B. afzelii* NE 632, and *B. garinii* of 84.67, 84.20, and 82.89°C, respectively, were obtained. Therefore, this PCR could differentiate *B. garinii* from *B. afzelii* and *B. burgdorferi* sensu stricto by its 1.3- to 1.8°C-lower T_m (Fig. 2c). These results confirm the data published by Pietila et al. (11).

Out of eight patient samples tested, only four gave positive results for amplification of the *recA* gene. Therefore, *recA* gene amplification is less sensitive in detecting borrelial DNA in skin samples than PCR amplification of the *p66* gene, which can be explained by the higher sensitivity generally yielded with nested PCR and with different DNA concentrations in the samples.

The *Borrelia* strains from patients 1, 2, 3, and 4 had mean T_ms in a range of 84.24 to 84.62°C. This result, in combination with the results obtained with the *p66* gene, would subtype them as *B. afzelii.* This result was confirmed by nucleotide sequencing of the *p66* amplicon (Fig. 1). Melting-curve analysis of the *p66* gene shows peaks for *B. burgdorferi* sensu stricto versus *B. garinii* and *B. afzelii*, whereas *recA* gene analysis could distinguish *B. garinii* from *B. afzelii* and *B. burgdorferi* sensu stricto; thus, the PCRs complement each other in subtyping different *Borrelia* species.

The choice of target is a crucial parameter for detecting and subtyping species within the *B. burgdorferi* sensu lato complex. The fragment of the *p66* gene (used here for the first time in LightCycler PCR, to our knowledge) has a wide heterogeneity in the three species and can be amplified in two steps with nested primers (14, 24). Molecular subtyping was performed with the same sequence of the *p66* gene by analysis of cRNA single-strand conformation polymorphisms (26). Interestingly, a published PCR protocol yielded greater sensitivities for most clinical samples using the *p66* nested primer set compared to another nested primer set targeting the plasmid gene *ospA* (12).

In conclusion, LightCycler nested PCR and melting-curve analysis of the *p66* gene enhance the sensitivity of detection of *B. burgdorferi* sensu lato DNA and are able to differentiate between melting peaks of *B. burgdorferi* sensu stricto and those of *B. afzelii*, which could not be separated by the previously reported amplification of the *recA* gene (11). The amplification of the two target genes, *p66* and *recA*, by LightCycler PCR and subsequent melting-curve analysis is a fast and reliable method to detect borrelial DNA in skin samples and to differentiate the three *Borrelia* species commonly associated with Lyme disease.

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