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In order to differentiate species within the Borrelia burgdorferi sensu lato complex, LightCycler 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 stricto (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 microorganisms 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 duplexes 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 Schwam (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 $T_m$ for these amplicons were calculated by the oligoapplet program available from TIB MOLBIOL, Berlin, Germany, which revealed
times 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* immunoglobulin 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 used.
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 MgCl₂.

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 continuously at every 0.1°C. For analysis of the melting curves, the LightCycler instrument’s software automatically converts them into melting peaks. The \( T_m \)s of the peaks were analyzed using the best-fit analysis software provided by Roche Molecular Biochemicals, and the mean \( T_m \)s 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 \( T_m \)s, 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).

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* from *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.
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 $T_m$s 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 $T_m$s, 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 $T_m$s 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 $T_m$s 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 $T_m$s 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.

REFERENCES


