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Distribution of Clinically Relevant *Borrelia* Genospecies in Ticks Assessed by a Novel, Single-Run, Real-Time PCR

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A LightCycler-based PCR protocol was developed which targets the *ospA* gene for the identification and quantification of the different *Borrelia burgdorferi* sensu lato species in culture and in ticks, based on the use of a fluorescently labeled probe (HybProbe) and an internally labeled primer. The detection limit of the PCR was 1 to 10 spirochetes. A melting temperature determined from the melting curve of the amplified product immediately after thermal cycling allowed the differentiation of the three different *B. burgdorferi* sensu lato genospecies (*B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*) that are clinically relevant in Europe in a single PCR run. This method represents a simplified approach to study the association of different *Borrelia* species in ticks, the risk of Lyme borreliosis, and the putatively species-specific clinical sequelae. To determine the reliability of the real-time PCR protocol, we studied the prevalence of *B. burgdorferi* sensu lato infection in *Ixodes ricinus* ticks. A total of 1,055 ticks were collected by flagging vegetation in five different sites in the region of Konstanz (south Germany) and were examined for the distribution of *B. burgdorferi* species by real-time PCR. The mean infection rate was 35%. Of 548 adult ticks, 40% were positive, and of 507 nymphs, 30% were positive. The predominant genospecies (with 18% mixed infections) in the examined areas was *B. afzelii* (53%), followed by *B. garinii* (18%) and *B. burgdorferi* sensu stricto (11%); 0.8% of the infecting *Borrelia* could not be identified.

Lyme borreliosis (LB)—the most common arthropod-borne infection in Europe (24) and the United States (25)—is a complex multisystem disorder caused by *Borrelia burgdorferi* sensu lato, a group of genetically diverse spirochetes. The principal vectors of these spirochetes are ticks belonging to the genus *Ixodes* (2).

The development of an erythema migrans rash at the site of the tick bite often characterizes the onset of LB. If left untreated, the infection can persist for years and may result in a range of clinical symptoms, which vary depending on the duration of the infection and the organs affected.

Isolates of *B. burgdorferi* sensu lato can be classified into different genomic species (1, 11). Only one of them, *B. burgdorferi* sensu stricto, has been implicated as the cause of disease in North America, but in Europe three genospecies, *Borrelia afzelii*, *Borrelia garinii*, and *B. burgdorferi* sensu stricto, are known to be pathogenic, and still others, such as *Borrelia valaisiana* and *Borrelia lusitaniae*, have been identified but are of unknown pathogenicity (7). Coinfections by two or more genomic groups of *B. burgdorferi* sensu lato have been found in ticks (13, 14) and patients with LB (5).

There is strong evidence that different species are involved in distinct clinical manifestations of the disease (28). Different studies have presented indirect evidence for the association of *B. garinii* with predominantly neurological symptoms (5), while infections by *B. burgdorferi* sensu stricto and *B. afzelii* tend to lead to arthritic symptoms (29) and cutaneous manifestations (3), respectively. New rapid and sensitive methods are therefore required for differentiating the three pathogenic *Borrelia* species to test the strengths of these associations.

PCR is increasingly employed for the detection of *Borrelia* (5, 17, 21, 23). Recently, the LightCycler PCR, which assesses the amount of amplified DNA after each PCR cycle, was introduced. Besides nonspecific DNA measurement by intercalating dyes (such as SYBR Green), specific gene probes labeled with fluorescent dyes allow the quantification of formed amplicon. Furthermore, calculation of the melting point of the DNA-probe adduct enables identification of the PCR product. This method can be exploited to distinguish sequence deviations, e.g., polymorphisms of different bacterial strains.

Recently, two LightCycler PCR-based assays for the differentiation of *Borrelia* species were described (15, 19). The second method, an amendment of the first, could distinguish among all three *Borrelia* species known to be pathogenic for humans. However, the method requires two LightCycler PCRs targeting the *recA* gene and the p66 gene, respectively. Melting curve analysis of the *recA* gene amplicon allows the separation of *B. garinii* from *B. burgdorferi* sensu stricto and *B. afzelii*, and melting curve analysis of the p66 gene amplicon is employed for the separation of *B. burgdorferi* sensu stricto from *B. afzelii* and *B. garinii*.

The LightCycler PCR described here allows rapid genotyping of the three *B. burgdorferi* species in a single PCR run. Therefore, a sequence of the *ospA* gene which results in zero, two, or three mismatches in the three species was chosen as a probe, allowing differentiation by melting point analysis. In contrast to the above-mentioned method of Mommert et al., a fluorescently labeled hybridization probe and an internally labeled primer were used rather than the fluorescent dye SYBR

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Green. This combination allows a rapid genotyping of the three *B. burgdorferi* species in a single PCR run.

In Europe, where all three genospecies of *Borrelia* known to be pathogenic for humans are found, the identification of the genospecies in patient specimens and ticks from patients is necessary to make a distinction among their respective roles in the pathophysiology of LB manifestations. Furthermore, field studies in areas of endemicity to assess the prevalence of *B. burgdorferi* sensu lato species in ticks will aid risk assessment and allow a determination of the infectivities of different species.

In the present study, we used real-time PCR to characterize the distribution of species of *B. burgdorferi* sensu lato in ticks collected in the field. We show that the three genomic groups were present in 1,055 *Ixodes ricinus* ticks collected in the southern part of Germany during 1999 and 2000 and that coinfections with two or three genomic groups of *B. burgdorferi* sensu lato occur among these ticks.

MATERIALS AND METHODS

Study area and tick collection. A total of 1,478 *I. ricinus* ticks were collected by the flagging method in five locations (A, B, H, L, and M) in the region of Konstanz (south Germany) in 1999 and 2000. The distances between the different locations were less than 10 km. At sites A and L, ticks were collected from the edges of roads and trails with mixed woodland on one side and meadows on the other side. Site B was a barbecue site in a forest situated near a marsh. M was a kindergarten in a forest, and at site H, ticks were collected from within the woodland near a path. The ticks were separated into nymphs and mature females and males and stored at −80°C until use. At least 200 ticks (100 nymphs, 50 males, and 50 females) from each location were examined by PCR.

Bacterial isolates and culture conditions. The *Borrelia* strains used in this study (*B. burgdorferi* sensu stricto N40, *B. garinii* PSth and B29, and *B. afzelii* VS461) were cultured in modified BSK-H medium (Sigma, Deisenhofen, Germany) at 33°C as described previously (6). All *Borrelia* strains were kindly provided by T. Kamradt (Berlin, Germany), with the exception of *B. garinii* A218, which was a gift from H. Martilla. Culture density was determined by microscopy using a modified Thoma counting chamber (Merck Eurolab, Ismaning, Germany).

DNA extraction. DNA from each *Borrelia* culture was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or by a Chelex-based method (31), which is a faster DNA extraction procedure. The bacterial culture was diluted 1:10 with 20% Chelex 100 (Bio-Rad Laboratories, Munich, Germany). After being thoroughly mixed, the sample was incubated at 56°C for 30 min. Then, the suspension was boiled for 10 min, and the debris was removed by centrifugation (13,800 × *g*; 3 min). The supernatant was either used directly for amplification or stored at −20°C until use. To extract the DNA from the ticks, they were mechanically crushed with sterile pestles, and nucleic acid extractions were performed by the Chelex-based method using 40 and 45 μl of Chelex 100 for nymphs and adults, respectively.

Real-time PCR. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany). A 296-bp fragment from the gene encoding OspA was amplified. The reverse primer used was published previously by Demaerschalck et al. (5). The forward primer, serving as the acceptor probe of the HybProbe detection system, was labeled internally with the fluorescent dye LC Red 640. Thus, first-strand DNA already contained fluorescent dye. For detection and differentiation of the species, we used a fluorescein-labeled probe. The forward primer was located further downstream than the originally published forward primer so that the fluorescein-labeled probe could bind the first-strand DNA close enough to the LC Red 640 for electron transfer to occur. The probe and primers were designed as shown in Fig. 1 and Table 1. The forward primer and probe were both designed by TIB Molbiol (DNA Synthesis Service, Roche Diagnostics, Berlin, Germany), who also synthesized all primers and the probe.

The 10-μl (final volume) PCR mixture included 1 μl of a commercial ready-to-use reaction mixture for PCR (LightCycler-DNA Fast Start master hybridization probes; Roche Diagnostics) that contains Hot Start *Taq* DNA polymerase, deoxynucleoside triphosphate mix, reaction buffer, and 1 mM $MgCl_2$. $MgCl_2$ was added to a final concentration of 5 mM. The final concentrations of the probe and the primers were 0.1 and 0.5 μM, respectively. Finally, 1 μl of template DNA was added.

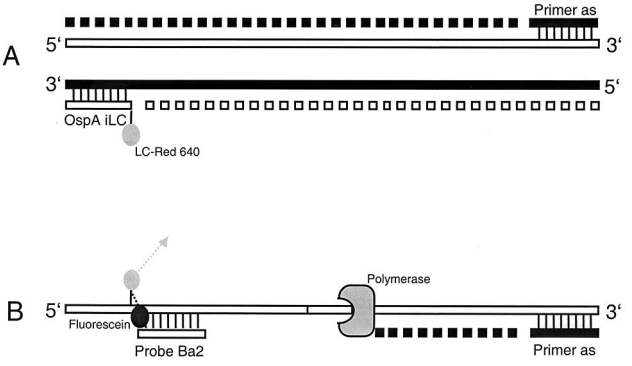

FIG. 1. Design of forward primer and probe for the identification of *Borrelia* species in the LightCycler. The forward primer (OspA iLC) labeled with LC Red 640 is incorporated into the first strand of DNA amplicon (A), and the fluorescein-labeled probe (Probe Ba2) binds to this DNA strand (B). The proximity of the two fluorochromes allows induction of LC Red 640 fluorescence by FRET. Due to the surplus of probes, the FRET signal depends directly on the amount of amplicon formed.

The reaction mixture was loaded into glass capillary tubes (Roche), which were snap sealed with plastic caps. The conditions for thermal cycling were as follows: initial denaturation for 10 min (to activate the fast-start *Taq* polymerase), followed by the amplification program, which included a denaturation step at 95°C for 10 s, an annealing step at 57°C for 10 s, and extension at 72°C for 13 s. Fluorescence was measured at the end of each annealing phase. The amplification was followed by a melting program, which started at 54°C for 45 s and then increased to 95°C at 0.1°C/s, with the fluorescence signal continuously monitored.

Agarose gel electrophoresis. PCR amplification products were resolved on 1.5% agarose gels by electrophoresis and visualized under UV light with ethidium bromide. As a marker, a 100-bp ladder was used (Gibco BRL, Karlsruhe, Germany). The expected amplification product was 296 bp.

Nested PCR and sequencing. Nested PCR targeting 5S-23S rRNA was performed by following the protocol of Rijpkema et al. (21).

The nucleotide sequences of PCR-amplified fragments were determined by the dideoxy chain termination technique (22) with the Prism Big Dye Terminator Cycle-Sequencing Ready-Reaction kit (Applied Biosystems) using the ABI Prism system 310 DNA sequencer.

Statistics. The Fisher test, an option of GraphPad (San Diego, Calif.) Instat, was used to determine statistical significance. A P value of ≤0.05 was considered significant.

RESULTS

Real-time PCR of *Borrelia* in culture samples. A total of 55 amplification cycles were performed with genomic DNA of each *Borrelia* genotype and a template-free control. The forward primer (internally labeled with LC Red 640) was incorporated into the first-strand DNA. After being annealed, the 3′

TABLE 1. Oligonucleotide primer and probe sequences used in PCR amplification and detection protocol of *B. burgdorferi* sensu lato strains

Primer or probe	Sequence[a]
Reverse primer	5′-CTA GTG TTT TGC CAT CTT CTT TGA AAA-3′
	5′-AGC CTT AAT AGC ATG C/TAA GCA AAA
Forward primer	X′TG-3′
Hybridization probe	5′-GCg CTG TTT TTT TCA TCA AGG CTG CTA ACX-3′

[a] X, fluorescein-labeled base; X′, LC Red 640-labeled base.

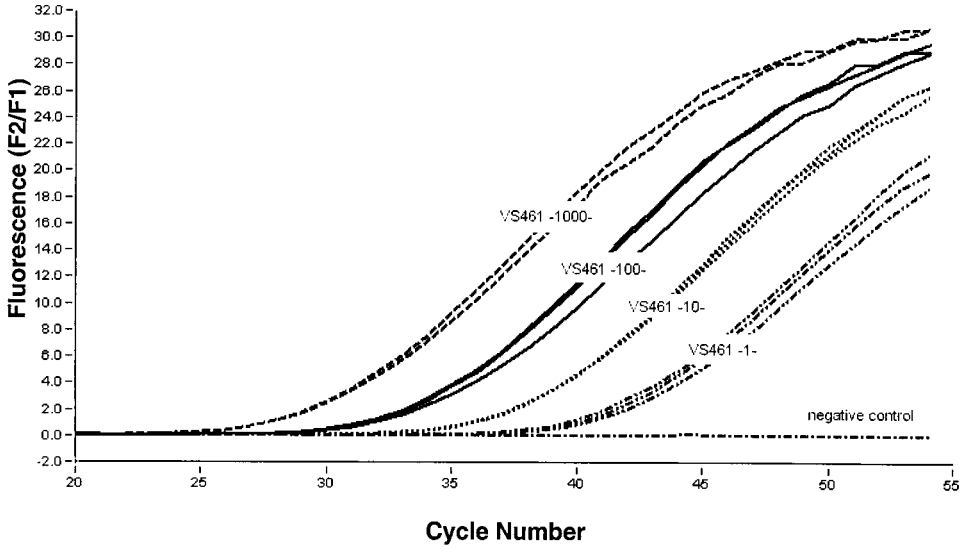

FIG. 2. Detection limit of PCR assay for detection of OspA determined with serial dilutions of VS461 genomic DNA. The dilutions were utilized in doublets or triplets. All samples were amplified simultaneously. The DNA in the sample corresponded to 1,000, 100, 10, and 1 spirochete of *B. afzelii* (VS461). As a negative control, water was added to the reaction mixture instead of template.

fluorescein-labeled probe bound to the first-strand DNA so that the fluorophores were separated by one base. This close proximity of the two dyes during hybridization allowed fluorescence resonance energy transfer (FRET) between the fluorophores. The resulting light emission of LC Red 640 was detected, providing real-time monitoring of the amplification process.

The fluorescence signal was measured at the end of each annealing phase and increased as the product accumulated in an exponential manner. No increase in fluorescence signal was observed in the absence of template. Figure 2 shows the progress of a PCR with Qiagen-extracted *Borrelia* DNA as the template. The DNA templates prepared by the Chelex method gave similar results (data not shown). The detection limit of the PCR was tested with serially diluted DNA templates of the three *Borrelia* strains from 10^8 *Borrelia* organisms/μl down to 10^0 *Borrelia* organisms/μl. It was found that a template DNA amount corresponding to 1 to 10 spirochetes (from strain N40 [data not shown], PSth [data not shown], or VS461 [Fig. 2]) was sufficient for detection. When uninfected ticks were spiked with known amounts of *Borrelia*, the recovery limit was also 1 to 10 spirochetes, despite attenuated PCR efficacy (slope of amplification), indicating the presence of inhibitors.

A cutoff criterion was defined based on the fluorescence variations of 60 uninfected ticks in 10 different LightCycler runs: the distribution of absolute fluorescence maxima of these 60 uninfected ticks was assessed, and the cutoff was defined as the mean ± 3 standard deviations, resulting in 0.5 F2/F1, i.e., the quotient of LC Red 640 to fluorescein signal. Ticks which did not reach 0.5 F2/F1 until cycle 55 were considered negative.

Real-time PCR allows the relative quantification of the amount of DNA template by using the cycle number at which the fluorescence signal starts to rise above a defined threshold (termed the ct value). In order to test the reproducibility of this measure, a *Borrelia* DNA standard was included in 55 independent LightCycler runs. The standard deviation was 1.32 cycles, showing excellent reproducibility of the method. The specificity of the PCR was confirmed by agarose (1.5%) gel electrophoresis (Fig. 3).

Since no reference method ("gold standard") is available and extraction of DNA from whole ticks excludes testing by methods other than PCR, the sensitivity and specificity of the assay cannot be assessed. In order to challenge the test and estimate these parameters, borderline positive-negative ticks were tested several times. Ticks (159) which were borderline in the first assay were analyzed repeatedly (a total of 410 measurements) until a definite classification was possible, i.e., the first result was either reproduced or falsified by repeated measurements. These data showed 3 false-negative (137 true-negative) and 8 false-positive (262 true-positive) measurements. From these data, a sensitivity of 98% and a specificity of 97% were estimated for this method.

Melting point analysis. After amplification, a melting curve was generated for genotyping, i.e., fluorescence was monitored continuously while the temperature was raised from 54 to 95°C. This resulted in a sharp decrease in LC Red 640 fluorescence at the temperature at which the probe dissociates from the template. When the strand and the probe are perfectly matched, strong hybridization results and the melting temperature (T_m) is high. A mismatch, even of a single base, results in a lower T_m, due to the decreased hybridization stability that is reflected in the peak of the first derivative of the melting curve ($-d\mathrm{F2}/dT$). Here, the probe was designed to match the sequence of the *Borrelia* species *B. afzelii* perfectly. The sequences of *B. garinii* and *B. burgdorferi* sensu stricto contained two and three mismatches with the probe, respectively. Therefore, a characteristic melting profile for each genotype was obtained.

The temperatures at which the probes detached from PCR products during the melting program were calculated using the LightCycler software. The T_ms of the three *Borrelia* species

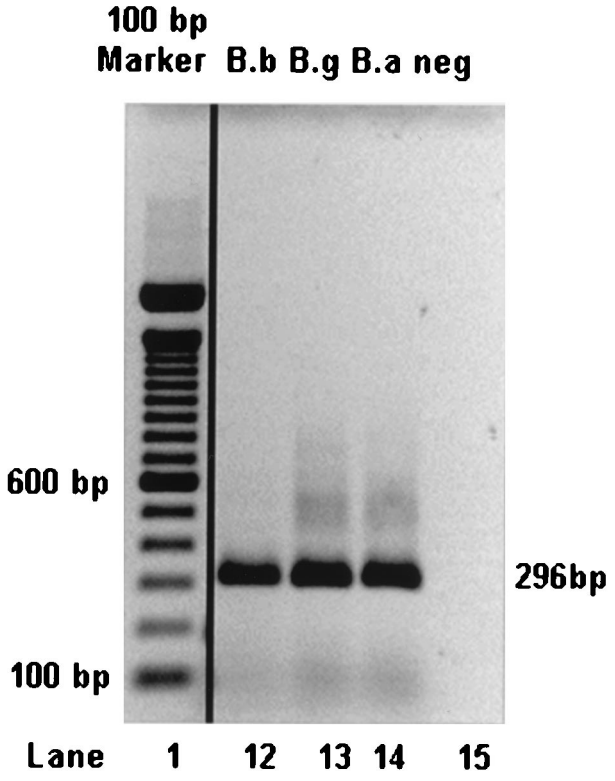

FIG. 3. Gel electrophoresis of PCR products from three *B. burgdorferi* sensu lato strains. The predicted lengths of the products were confirmed on a 1.5% agarose gel. A 100-bp ladder was used as a size marker (first lane). The other lanes show PCR products obtained from *B. burgdorferi* sensu stricto (B.b; lane 12), *B. garinii* (B.g; lane 13), and *B. afzelii* (B.a; lane 14) and a water control (neg; lane 15).

differed, as shown in Fig. 4. The average T_ms of the reference strains of *B. burgdorferi* sensu stricto (N40), *B. garinii* (PSth), and *B. afzelii* (VS461) were 63, 68, and 72.5°C, respectively. Thus, the difference between the T_ms of N40 and PSth was 5°C and the difference between those of PSth and VS461 was more than 4°C. As with this method each T_m corresponded specifically to one *Borrelia* species, we were also able to detect the different species in mixtures of two or three *Borrelia* genospecies (Fig. 5). Two criteria were used to define a positive signal in T_m analysis: (i) a relative maximum (i.e., a peak, to be distinguished from a plateau) of fluorescence at the characteristic temperature and (ii) a height of this peak of at least 10% of the peak of the control (representing about 100 *Borrelia* organisms). This procedure resulted in a cutoff around 0.3 $-dF2/dT$.

Detection of *B. burgdorferi* sensu lato in ticks by real-time PCR. The reliability of the real-time PCR protocol for the amplification of *B. burgdorferi* OspA was tested by assessing the infection rate in ticks collected in the field (Table 2). A total of 1,055 *I. ricinus* ticks collected at five different sampling sites in the region of Konstanz were investigated by real-time PCR. As summarized in Table 2, the infection rates for the ticks examined at the different sites varied significantly from 20 to 57% ($P < 0.001$). The overall prevalence of *B. burgdorferi* sensu lato in ticks was 35%. The highest infection rate (57%) was found in location B. Further, the overall infection rates in nymphs were significantly ($P < 0.001$) lower (30%, i.e., 152 out of 507) than those in the adult ticks (40%, i.e., 219 out of 548).

An estimation of *Borrelia* numbers in ticks was carried out employing the ct values of the individual runs. In spike experiments, 1,000 *Borrelia* genome equivalents corresponded to a ct value of 30, 100 genome equivalents corresponded to 34, 10 genome equivalents corresponded to 38, and 1 genome equivalent corresponded to more than 40. The distribution of ct values of all 371 positive ticks is shown in Fig. 6, with a median of 34, i.e., about 4,000 *Borrelia* organisms per tick.

Genotyping (Table 3) indicated that *B. afzelii* was the predominant species in all of the areas studied. *B. afzelii* was detected in 70% of the infected ticks, followed by *B. garinii* (34%) and *B. burgdorferi* sensu stricto (12%). The *Borrelia* species infecting three ticks (0.8%) could not be identified, since they showed a T_m of 58.6°C, which did not correspond to any T_m of the other species. Mixed infections by two or three species were detected in 18% of the ticks characterized as positive by PCR. We detected double infections of *B. afzelii* with *B. garinii* (88%) or *B. burgdorferi* sensu stricto (9%). In one case, we could detect a mixed infection of all three species. A combination of *B. garinii* and *B. burgdorferi* sensu stricto alone was not found.

The PCR products of 24 *Borrelia* isolates from ticks were sequenced to determine the genospecies. All 10 *B. afzelii*, 10 *B. garinii*, and 4 *B. burgdorferi* sensu stricto isolates showed the expected number of mismatches with the probe, stressing the validity of genotyping by melting point analysis. However, two well-characterized strains of *B. garinii* (B29 and A218) resulted in two peaks at 68 and 72.5°C in the melting point analysis, which would falsely indicate a double infection with *B. garinii* and *B. afzelii*. Sequencing of these strains did not clarify the occurrence of the two peaks, since both strains displayed the two mismatches only with the probe characteristic for *B. garinii*. Nevertheless, this finding might indicate that the frequency of double infection (5.6% of all ticks) with *B. garinii* and *B. afzelii* is overestimated. However, assuming independence of both infections, a very similar frequency of double infections of 3% can be calculated from the frequencies of either infection alone.

DISCUSSION

The aim of our study was the development of a new PCR method for the detection and differentiation of the *B. burgdorferi* sensu lato species *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto. The use of the LightCycler system allowed the simultaneous differentiation of these species in one PCR run in a single tube, representing a much faster, less laborious, and less expensive method for genotype identification than the commonly used methods, including species-specific PCR, randomly amplified polymorphic DNA analysis, PCR-based sequencing, and restriction fragment length polymorphism.

The *ospA* gene was used as the target of real-time PCR. This gene is located on a linear 49-kb plasmid. The sequences of the *ospA* genes of the three major *Borrelia* species are different (33): *ospA* genotypes 1 and 2 correspond to *B. burgdorferi* sensu stricto and *B. afzelii*, respectively, and *ospA* genotypes 3 to 7 correspond to *B. garinii*. These genotypes correspond to the OspA serotypes 1 to 7 (34). Due to the hypothesized multiplicity of plasmid genes in clinical probes (18), portions of the

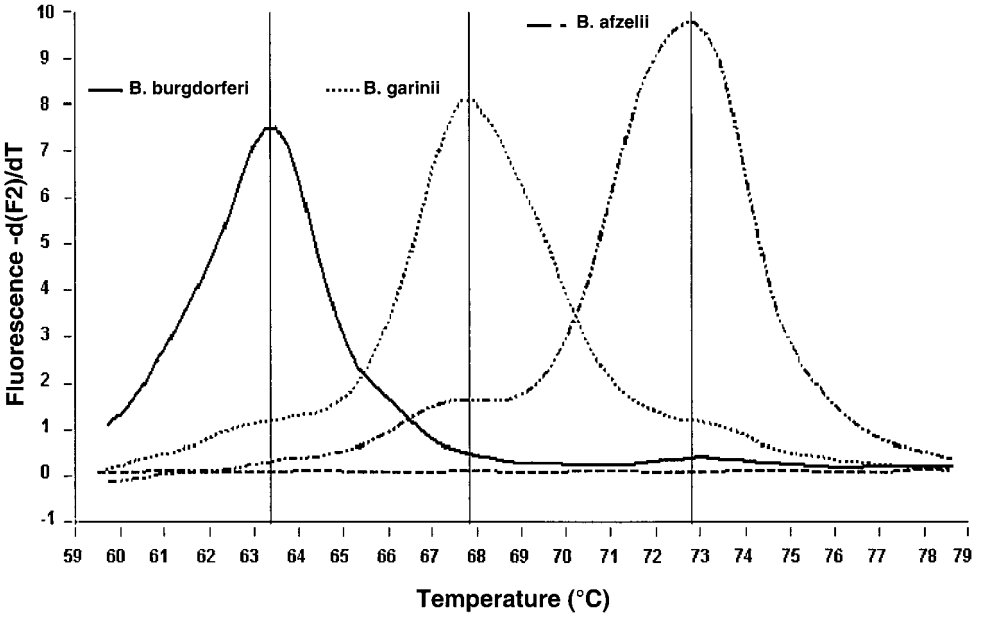

FIG. 4. Identification of T_ms for the three *B. burgdorferi* sensu lato species. The melting peak analysis which followed each PCR run showed that the T_ms were 63°C for *B. burgdorferi* sensu stricto (N40), 68°C for *B. garinii* (PSth), and 72.5°C for *B. afzelii* (VS461).

ospA gene were frequently chosen as templates for PCR (5, 8, 16, 30).

A study by Will et al. (33) showed highly conserved *ospA* genes within the *B. burgdorferi* sensu stricto group as well as within the *B. afzelii* group but heterogeneity within the *ospA* genes of *B. garinii*-type strains. In line with this notion, a BLAST search in the National Center for Biotechnology Information (GenBank) databases (22 *B. garinii* sequences) for the *B. garinii ospA* sequence binding to the probe showed two distinct groups of *B. garinii*, each characterized by two mismatches with our probe (at positions 1 and 6 and positions 1 and 12, respectively). All 10 *B. burgdorferi* sensu stricto and 16 *B. afzelii* sequences showed zero or three mismatches, respectively. Since the method is extremely sensitive to changes in the sequence binding to the probe, misclassifications cannot be completely excluded.

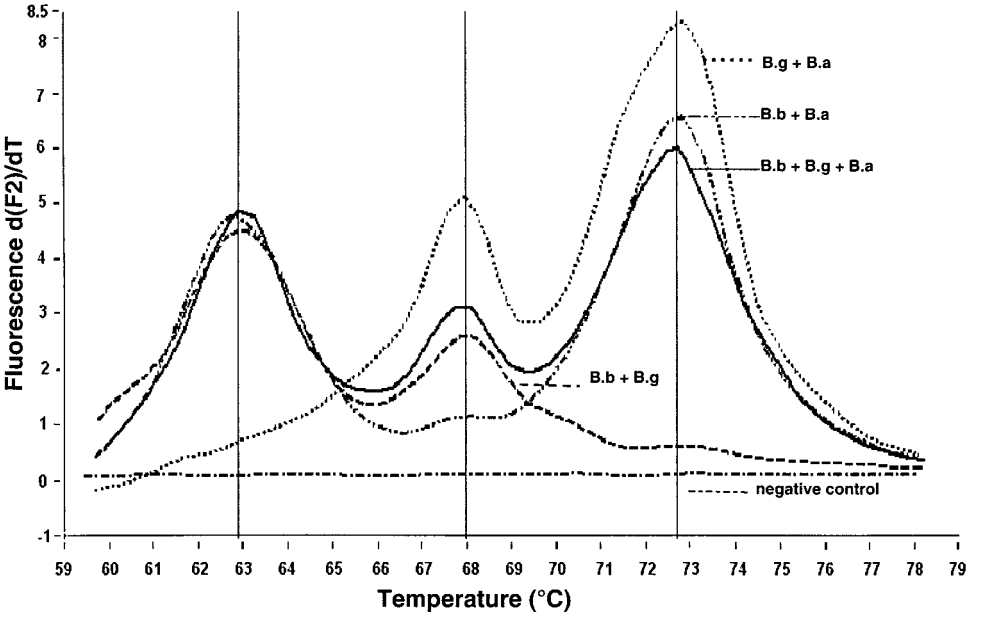

FIG. 5. Melting point analysis of mixtures of *Borrelia* species. Differentiation of species in coinfections with two (*B. afzelii* [B.a] VS461 plus *B. garinii* [B.g] PSth, VS461 plus *B. burgdorferi* sensu stricto [B.b] N40, or N40 plus PSth) or three (N40 plus PSth plus VS461) *Borrelia* species. In the negative control, water was used as a template.

TABLE 2. Summary of *Borrelia* infection rates in different maturation stages of *I. ricinus* ticks from different collection sites

Tick	Infection rate											
	A[a]		B		H		L		M		Σ	
	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%
Female	15/50	30	50/83	60	10/40	25	17/50	34	11/52	21	103/275	37
Male	18/50	36	43/67	64	16/56	29	18/60	36	21/50	42	116/273	42
Adult	33/100	33	93/150	62	26/96	27	35/100	35	32/102	31	219/548	40
Nymph	27/100	27	53/106	50	14/100	14	36/100	36	22/101	22	152/507	30
Σ	60/200	30	146/256	57	40/196	20	71/200	36	54/203	27	371/1,055	35

[a] Five different collection sites in Konstanz. +, positive.

The PCR assay described here is sensitive enough to detect fewer than 10 spirochetes of each of the three clinically relevant genospecies of *B. burgdorferi* sensu lato in a sample. Therefore, the detection limit is comparable to that of conventional nested PCRs. However, the real-time PCR is less laborious and considerably faster. The entire assay can be completed in approximately 1 h. Amplification, hybridization, and analysis are performed in one closed capillary tube, decreasing the risk of cross contamination. The melting curve analysis allows the differentiation of *Borrelia* species even in a mixture of all three species. Thus, the method presented here appears to be the first LightCycler-based PCR which allows the differentiation of the three *Borrelia* species *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* in one PCR run.

Since any naturally occurring tick can be analyzed by only a single type of procedure and no gold standard for *Borrelia* detection is available, it was only possible to compare the new method to an established nested PCR in a block cycler targeting 5S-23S rRNA (21). Of 100 positive ticks, 86 were also positive in nested PCR, while all 50 negative ticks were negative in both assays. Taking nested PCR as the reference method, this would indicate a sensitivity of 100%, a specificity of 86%, and accuracy of 91%.

The observation that 14 ticks were positive only by real-time PCR might indicate either a higher sensitivity of the LightCycler or false-positive results. Employing serial dilutions of *Borrelia* DNA, no major difference in the limit of detection was observed. However, the nested PCR might contain higher concentrations of tick-borne inhibitors due to the larger amounts of tick extract used per tube (10 versus 20% of total volume).

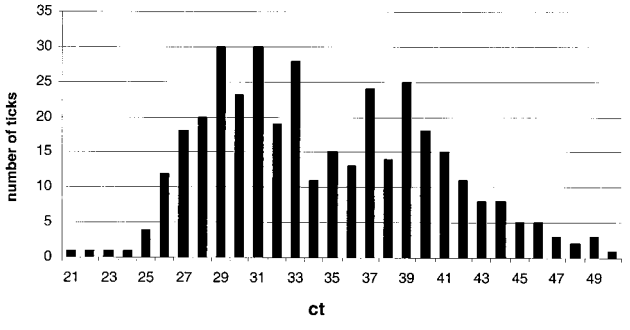

FIG. 6. Distribution of *Borrelia* counts in all 371 positive ticks. The cycle number at which the PCR fluorescence commences (ct value) allows the relative quantification of the number of genome equivalents present in the probe. Dilution series of *Borrelia* DNA showed that 1,000 *Borrelia* genome equivalents corresponded to a ct value of 30, 100 genome equivalents corresponded to 34, 10 genome equivalents corresponded to 38, and 1 genome equivalent corresponded to more than 40 cycles.

There is an ongoing discussion about the clinical relevance of *B. valaisiana* and *B. lusitaniae*, which are occasionally found in ticks and birds (7). Due to sequence similarity, *B. valaisiana* yields the same melting point as *B. afzelii* in our system and thus cannot be distinguished. A total of 39 tick samples classified as *B. afzelii* infected were therefore subjected to Southern blot analysis according to the method described previously (21). Only a single case of *B. valaisiana* infection was found (data not shown), indicating that the prevalence of this genotype is very low in the investigated area.

The sensitivity and specificity of our method were estimated to be 98 and 97%, respectively, based on 159 ticks, which were analyzed two to six times. It is worth noting that ticks with low and borderline bacterial burdens were selected to challenge the method, thus actually underestimating the reproducibility in practice.

To test the feasibility and reliability of the real-time PCR protocol for the amplification of *B. burgdorferi ospA*, we investigated the distribution of *B. burgdorferi* sensu lato in ticks collected in Konstanz and determined the genomic groups present. Information about the prevalence of *Borrelia* infection in ticks in areas of endemicity is necessary for risk assessment. It has been shown that most habitats where ticks carrying *Borrelia* have been found are recreational sites (10). Therefore, we selected four sites with recreational functions within the five collection sites. Significant variability was observed in the prevalence of *B. burgdorferi* sensu lato among the sites examined, with infection rates ranging from 20 to 57%. These considerable differences in the prevalence of infected ticks even in habitats in close proximity indicates that the occurrence of *Borrelia* species in nature is affected by many ecological factors.

A very high infection rate (57%) was found at one of the five sites. Similar results showing high infection rates in particular sites have also been described by others (4, 12, 20). One possible explanation for this phenomenon could be that the transmission of the spirochetes to ticks is amplified by cofeeding on a vertebrate host, as has been proposed (9).

By using melting point analysis, we were able to differentiate the three genospecies of *Borrelia* known to be pathogenic for humans in the ticks collected. All three genomic groups (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) were found in tick isolates from the investigated sites. Our findings indicate

TABLE 3. Identification of different *B. burgdorferi* sensu lato species in *I. ricinus* ticks collected at five different sites (A, B, H, L, and M) in Konstanz

Species[a]	Infection rate											
	A		B		H		L		M		Σ	
	No. +[b]	%	No. +	%	No. +	%	No. +	%	No. +	%	No. +	%
B.b.	7	12	16	11	7	18	0	0	9	17	39	11
B.g.	20	33	20	14	7	18	18	18	6	11	66	18
B.a.	23	38	80	55	18	45	46	65	28	52	195	53
Unknown	0	0	2	1	0	0	2	3	0	0	4	1
B.b. + B.g.	0	0	0	0	0	0	0	0	0	0	0	0
B.b. + B.a.	0	0	2	1	0	0	1	1	3	6	6	2
B.g. + B.a.	9	15	26	18	8	20	9	18	7	13	59	16
B.a + unknown	0	0	0	0	0	0	0	0	1	2	1	0.3
B.b + B.g + B.a	1	2	0	0	0	0	0	0	0	0	1	0.3
Total positive	60	30	146	57	40	20	71	36	54	27	371	35
Total measured	200		256		196		200		203		1,055	

[a] B.b., *B. burgdorferi* sensu stricto; B.g., *B. garinii*; B.a., *B. afzelii*.
[b] +, positive.

that *B. afzelii* (70%) was present most abundantly, followed by *B. garinii* (34%). *B. burgdorferi* sensu stricto (12%) appears to be less common here. Similar findings were made in Slovenia, where out of 60 *Borrelia*-positive ticks, 53% were identified as infected by *B. afzelii*, 33% were identified as infected by *B. garinii*, and only 13% were identified as infected by *B. burgdorferi* sensu stricto (26).

Interestingly, three of the 1,055 ticks examined were positive for *B. burgdorferi* sensu lato but the isolates could not be classified into one of the three species, as their T_m (58.6°C) differed from those of the others: *B. burgdorferi* sensu stricto (63°C), *B. garinii* (68°C), and *B. afzelii* (72.5°C). A DNA sequence analysis (performed by MWG Biotech, Ebersberg, Germany) indicated that these spirochetes belong to the recently described *Borrelia* species strain A14S (32). A14S is phenotypically and genetically different from all other *B. burgdorferi* sensu lato species described and therefore most likely represents a new *Borrelia* genotype. Since it was cultured from a skin biopsy specimen of a patient with erythema migrans, it seems to be pathogenic for humans.

Mixed infections were found in 18% of the *Borrelia*-positive ticks—mainly double infections by *B. afzelii* and *B. garinii*. Double infections by *B. garinii* and *B. burgdorferi* sensu stricto were not found.

The quantification of *Borrelia* species in ticks showed a very heterogeneous distribution ranging from 1 to more than 1,000 *Borrelia* equivalents. Similar numbers were found by Stünzner et al. by microscopically counting *Borrelia* organisms in tick guts (27). This finding illustrates that a method with a low detection limit is required to assess all infected ticks. To our knowledge, the impact of different *Borrelia* burdens on infectivity for mammals has not yet been studied.

In conclusion, we have demonstrated that the use of the new real-time PCR method provides a rapid and sensitive tool for differentiating *B. burgdorferi* sensu lato species known to be pathogenic for humans. We have shown that the method can be applied for the detection and differentiation of *Borrelia* genospecies in ticks collected in the field and ticks removed from humans. The novel method appears to represent a versatile tool to assess the roles of different genospecies in the pathophysiology of Lyme disease in Europe.

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