Real-Time PCR for Simultaneous Detection and Quantification of *Borrelia burgdorferi* in Field-Collected *Ixodes scapularis* Ticks from the Northeastern United States

Guiqing Wang,¹ Dionysios Liveris,¹ Brandon Brei,^{2†} Hongyan Wu,¹ Richard C. Falco,³
Durland Fish,² and Ira Schwartz^{1,4*}

Department of Microbiology and Immunology¹ and Department of Medicine,⁴ New York Medical College, Valhalla, New York 10595; Department of Epidemiology and Public Health, Yale School of Medicine, New Haven, Connecticut 06520²; and Vector Ecology Laboratory, Calder Ecology Center, Fordham University, Armonk, New York 10504³

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The density of spirochetes in field-collected or experimentally infected ticks is estimated mainly by assays based on microscopy. In this study, a real-time quantitative PCR (qPCR) protocol targeting the *Borrelia burgdorferi*-specific *recA* gene was adapted for use with a Lightcycler for rapid detection and quantification of the Lyme disease spirochete, *B. burgdorferi*, in field-collected *Ixodes scapularis* ticks. The sensitivity of qPCR for detection of *B. burgdorferi* DNA in infected ticks was comparable to that of a well-established nested PCR targeting the 16S-23S rRNA spacer. Of the 498 *I. scapularis* ticks collected from four northeastern states (Rhode Island, Connecticut, New York, and New Jersey), 91 of 438 (20.7%) nymphal ticks and 15 of 60 (25.0%) adult ticks were positive by qPCR assay. The number of spirochetes in individual ticks varied from 25 to 197,200 with a mean of 1,964 spirochetes per nymphal tick and a mean of 5,351 spirochetes per adult tick. No significant differences were found in the mean numbers of spirochetes counted either in nymphal ticks collected at different locations in these four states ($P = 0.23$ by one-way analysis of variance test) or in ticks infected with the three distinct ribosomal spacer restriction fragment length polymorphism types of *B. burgdorferi* ($P = 0.39$). A high degree of spirochete aggregation among infected ticks (variance-to-mean ratio of 24,877; moment estimate of $k = 0.279$) was observed. From the frequency distribution data and previously published transmission studies, we estimated that a minimum of 300 organisms may be required in a host-seeking nymphal tick to be able to transmit infection to mice while feeding on mice. These data indicate that real-time qPCR is a reliable approach for simultaneous detection and quantification of *B. burgdorferi* infection in field-collected ticks and can be used for ecological and epidemiological surveillance of Lyme disease spirochetes.

Hard ticks (Ixodidae) in the *Ixodes persulcatus* complex, such as *Ixodes scapularis* and *Ixodes pacificus* in North America, *Ixodes ricinus* in Europe, and *Ixodes persulcatus* in far eastern Russia and Asia, are the principal vectors of the Lyme disease spirochete, *Borrelia burgdorferi*, and several other tick-borne pathogens (2, 25). The incidence of human Lyme disease in areas where it is endemic is correlated with the abundance and prevalence of *Ixodes* ticks infected with *B. burgdorferi* (34). Moreover, studies of laboratory animals have suggested that the efficiency of host infection is determined in part by the number of spirochetes inoculated using a needle or deposited by the tick at the time of feeding (21, 27). Thus, monitoring *B. burgdorferi* density in host-seeking ticks will provide more accurate data for assessment of population risk of Lyme disease and the potential variability of disease manifestations after tick bites.

Currently, the number of spirochetes in field-collected or experimentally infected *Ixodes* ticks is estimated mainly by microscopy-based assays in which the spirochetes are counted directly on tick midgut smears stained with fluorescently labeled specific antibodies (8, 9, 27) or on silver-stained histo-

logical sections of ticks (11, 14). For specific detection of *B. burgdorferi* in field-collected adult *I. scapularis* ticks and monitoring of the spirochete kinetics during the tick life cycle, an OspA antigen-capture enzyme-linked immunosorbent assay (ELISA) was also developed (3–5). These methods are labor-intensive, which limits the number of ticks that can be analyzed. Moreover, the sensitivities of these methods are relatively low, e.g., a lower detection limit of approximately 150 spirochetes was reported for the antigen-capture ELISA (5).

Real-time PCR has features that allow for rapid detection of infectious pathogens in environmental or experimentally infected animal tissues and clinical specimens (38). The technique has recently been employed to detect the presence of *B. burgdorferi* DNA in *I. ricinus* ticks from Switzerland (15), to quantify the spirochete loads in experimentally infected animal tissues (23, 24, 35), and to differentiate the three *B. burgdorferi* sensu lato species that are pathogenic to humans in Europe (22, 28, 30). By targeting the *B. burgdorferi*-specific *recA* gene, we have successfully developed a real-time PCR protocol to quantify the spirochetes in infected animal tissues and in skin biopsy specimens of patients with erythema migrans (19, 36, 37). The present study evaluates a modified real-time, quantitative PCR (qPCR) protocol for simultaneous detection and quantification of *B. burgdorferi* DNA in field-collected *I. scapularis* ticks from the northeastern United States. The genotypic

* Corresponding author. Mailing address: Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595. Phone: (914) 594-4658. Fax: (914) 594-4176. E-mail: schwartz@nymc.edu.

† Deceased.

FIG. 1. Distribution of spirochete burdens in 91 *B. burgdorferi*-positive *I. scapularis* nymphs from the northeastern United States. The spirochete burdens were determined by a real-time qPCR. (A) Before data transformation, the distribution is skewed to the left and overdispersed. (B) After data transformation, the distribution is normal. The dark gray bars indicate the 18.4% of ticks with the fewest (<300) spirochetes.

distribution of *B. burgdorferi* in host-seeking ticks and the spirochete loads of ticks infected with distinct genotypes of *B. burgdorferi* are also analyzed.

MATERIALS AND METHODS

Tick collection. *I. scapularis* ticks were collected from four northeastern states (New York, Connecticut, Rhode Island, and New Jersey) of the United States by drag sampling as previously described (6, 32). A total of 292 *I. scapularis* ticks (232 nymphs and 60 adults) were collected from five different locations in the lower Hudson valley of New York during 1998 and 2000. An additional 206 nymphal *I. scapularis* ticks collected from southeastern Rhode Island ($n = 61$), southern Connecticut ($n = 81$), and eastern New Jersey ($n = 64$) for a separate project in 1999 were also included in this study.

Preparation of DNA from ticks. All ticks were preserved in vials containing 70% ethanol until DNA extraction. DNA was prepared from ticks by using a commercial DNA extraction kit (Isoquick; Orca Research, Bothell, Wash.) as previously described (31). This method effectively removes potential PCR inhibitors from blood-fed ticks (31). Extracted DNA was resuspended in 50 μ l of sterile water. Five and two microliters of such DNA were used for nested PCR and real-time PCR, respectively.

Real-time qPCR. Simultaneous detection and quantification of *B. burgdorferi* DNA in ticks were performed with the Lightcycler PCR instrument (Roche Diagnostics, Mannheim, Germany), for which the *B. burgdorferi*-specific chromosome-encoded *recA* gene was chosen as the target. It is assumed that only a single copy of *recA* is present per spirochete based on the genome sequence of *B. burgdorferi* type strain B31 (10) and a previous experimental report (23). qPCR was performed in 10- μ l reaction mixtures containing 1 $\times$ FastStart Taq DNA polymerase mixture (Roche), 4 mM MgCl₂, 1 μ M concentrations of primers 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') (23), and 2 μ l of tick DNA extract. An external standard template containing 10 to 10⁵ copies of the *B. burgdorferi* *recA* gene was included in each run to generate a standard curve. The amplification program was performed as follows: (i) heating at 95°C for 10 min to activate the FastStart Taq polymerase; (ii) 45 cycles, with 1 cycle consisting of increasing the temperature 20°C/s to 95°C and holding the temperature at 95°C for 10 s, decreasing the temperature 20°C/s to 60°C and holding the temperature at 60°C for 5 s, and increasing the temperature 20°C/s to 72°C and holding the temperature at 72°C for 10 s. The fluorescent product was collected at 82°C at the last step of each cycle to minimize signal from nonspecific products. A melting curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 60°C, and slowly heating it at 0.2°C/s to 95°C with fluorescence collection at 0.2°C intervals. The number of spirochetes in each PCR mixture was calculated by comparing the crossing points of the samples with those of the standards with the Lightcycler software. The spirochete load in each tick was estimated by multiplying the number of spirochetes determined in each PCR mixture by a factor of 25 (since only 2 μ l of a

50- μ l DNA extract was used per reaction mixture). The specificity of qPCR products was confirmed by melting curve analysis and/or gel-based post-PCR analysis. A sample was considered positive only if it exhibited a log-linear (exponential) phase of amplification in the fluorescence curve and either it showed a specific peak with the melting temperature at 85°C in the melting curve analysis or, if post-PCR analysis was performed, a specific band with the expected size was visible on a 1.5% agarose gel.

PCR amplification and RFLP analysis. A nested PCR which amplifies a portion of the 16S-23S ribosomal DNA spacer of *B. burgdorferi* was performed as previously described (18). The primers for the first round of PCR were P_A (5'-GGTAT GTTGA GTGAG GG-3') and P₉₅ (5'-GGTTA GAGCG CAGGT CTG-3') and would yield a 1,014-bp product on the basis of the sequence of isolate B31 (10). Two microliters of a 1/100 dilution of the first-round PCR product was employed as the template in the second round of PCR with primers P_B (5'-CGTAC TGGAA AGTGC GGCTG-3') and P₉₇ (5'-GATGT TCAAC TC ATC CTGGT CCC-3'). Positive samples yielded a DNA fragment with a size of approximately 940 bp that was subjected to restriction fragment length polymorphism (RFLP) analysis by digestion with *Mse*I as described elsewhere (17, 18).

Statistical analysis. Quantitative data obtained by real-time qPCR were analyzed using Minitab (release 12; South College, Pa.) and Excel97 (Microsoft Corp., Redmond, Wash.) software. To examine the distribution of *B. burgdorferi* in infected ticks, a histogram of spirochete counts from the PCR-positive nymphs ($n = 91$) was generated (Fig. 1A). As the distribution is skewed to the left and overdispersed, the data were transformed to normally distributed data with a $\ln(x + 10)$ transformation (Fig. 1B) for parametric statistical analysis. A Kolmogorov-Smirnov test was applied to verify that the transformed data satisfied the assumption of normal distribution. The degree of spirochete aggregation among both infected host-seeking ticks and all host-seeking ticks was quantified by calculating the variance-to-mean ratios (s^2/m) and the corrected moment estimates of k [$k = (m^2 - s^2/n)/(s^2 - m)$] (12).

To determine whether the prevalence of *B. burgdorferi* infection differed in nymphs in the geographic locations sampled, the percentage of ticks infected from each of the eight collection locations was computed, a one-way analysis of variance (ANOVA) was then performed by Tukey's method to detect significant differences in the prevalence of tick infection in different locations. ANOVA using transformed data was also employed to determine whether the spirochete load in infected ticks is correlated with geographic location or ribosomal spacer restriction fragment length polymorphism type (RST) genotype. For all ANOVA comparisons, the normality of the data (counts) was evaluated with a Kolmogorov-Smirnov test or binomial data were examined for central limit theorem violations. We also evaluated variance equalities with Bartlett's test. For reporting data, the mean number of spirochete loads and 95% confidence intervals were back transformed. The spirochete numbers present in the 18.4% of infected ticks with the lowest numbers of spirochetes, which corresponds to the reported proportion unable to transmit in a previously published laboratory study (16), was used to estimate a threshold number of spirochetes needed to transmit infection from infected tick to reservoir host.

TABLE 1. Prevalence of *B. burgdorferi* infection and spirochete loads in field-collected *I. scapularis* ticks from northeastern United States

Tick stage and geographic location	No. of ticks tested	No. of ticks infected (%)	Spirochete load in infected ticks ^a		
			Median	Mean	95% CI
Nymphs					
Rhode Island	61	9 (14)	1,500	1,011	123–7,846
Connecticut	81	24 (30)	3,388	1,759	729–4,229
New York sites					
Armonk	99	23 (23)	3,300	2,522	330–18,815
New Rochelle	17	12 (71)	3,325	3,489	1,064–11,394
Rye	26	0 (0)			
White Plains	40	8 (20)	1,588	2,424	732–7,977
Yonkers	50	4 (8)	7,400	4,988	1,550–16,004
Total	232	47 (17)	3,200	2,795	1,738–4,491
New Jersey	64	11 (17)	1,675	949	340–2,621
Total	438	91 (21)	2,925	1,964	1,329–2,900
Adults					
New York	60	15 (25)	5,750	5,351	2,470–11,592

^a The median numbers of spirochetes in infected ticks are based on original data from real-time qPCR. Means and 95% confidence intervals (95% CI) were back transformed.

RESULTS

Detection and quantification of *B. burgdorferi* in field-collected ticks. A total of 498 field-collected *I. scapularis* ticks from four northeastern states were tested by qPCR for the presence of *B. burgdorferi*-specific DNA. Ninety-one of 438 (20.7%) nymphal ticks and 15 of 60 (25.0%) adult ticks were positive ($P > 0.05$). The number of spirochetes in individual ticks varied from 25 to 86,750 per infected nymph and from 350 to 197,200 per infected adult tick. The mean numbers of spirochetes were 1,964 per infected nymphal tick and 5,351 per infected adult tick ($P = 0.05$). No significant differences in the prevalence of *B. burgdorferi* infection in host-seeking *I. scapularis* nymphs on the basis of collection location were detected ($P = 0.23$; $df = 4$; $n = 345$) (Table 1). Ticks from three sites in New York (New Rochelle, Rye, and Yonkers) were excluded from this analysis because these samples did not satisfy central limit theorem assumptions. The differences in the spirochete loads of infected nymphal ticks from different locations were not significant ($P = 0.50$; $df = 6$; $n = 91$).

Spirochete aggregation and threshold to transmit infection.

Of the 438 nymphal *I. scapularis* ticks analyzed in this study, the untransformed arithmetic mean *B. burgdorferi* counts (used to quantify aggregation) were 7,216 spirochetes in infected ticks ($n = 91$) and 1,499 spirochetes in all ticks ($n = 438$). There was a high degree of spirochete aggregation among infected ticks ($s^2/m = 24,877$; $k = 0.279$) and extreme spirochete aggregation among all ticks ($s^2/m = 30,390$; $k = 0.047$). A previous experiment showed that only 81.6% (31 of 38) of nymphal ticks infected with *B. burgdorferi* transmitted infection to laboratory mice (16). The spirochete numbers present in the 18.4% of infected ticks with the lowest number of spirochetes, which corresponds to the proportion unable to transmit infection was used to estimate a threshold number of spirochetes needed to transmit infection from tick to reservoir host. As

shown in Fig. 1B, the 18.4% of infected ticks with the lowest spirochete burdens harbored ≤ 300 spirochetes each.

Spirochete loads in ticks infected with distinct genotypes of *B. burgdorferi*. We have previously reported the typing of *B. burgdorferi* based on PCR-RFLP of the 16S-23S ribosomal DNA intergenic spacer (13). This approach was employed to assess the genetic diversity among *B. burgdorferi* sensu stricto isolates from patients with early Lyme disease; all clinical isolates were classified into three genetically distinct groups, designated RST1, RST2, and RST3 (13, 17, 18). In this study, nested PCR amplification and subsequent RFLP analysis of the 16S-23S rRNA spacer amplicon was performed on 348 of 498 (69.9%) *I. scapularis* ticks. The distribution of *B. burgdorferi* genotypes and the spirochete load in 73 infected ticks for which both genotyping and quantitative data were available are summarized in Table 2. Twenty-six percent (19 of 73) of the ticks were infected with the RST1 genotype of *B. burgdorferi*, 20.6% (15 of 73) were infected with RST2, and 39.7% (29 of 73) were infected with RST3; 13.7% of positive ticks had mixed infection of two or more genotypes. The percentage of ticks infected with the RST3 genotype was higher than those infected with RST1 ($P = 0.07$) and RST2 ($P = 0.01$) genotypes. However, no significant difference was found in the mean number of spirochetes in ticks infected with distinct genotypes of *B. burgdorferi* ($P = 0.39$; $df = 2$; $n = 63$).

Comparison of nested and real-time qPCR. First, both nested PCR and qPCR were performed on DNA isolated from 348 ticks to determine the relative sensitivities of the methods. Later, another 150 ticks were added to increase the sample size from underrepresented locations for qPCR comparisons among geographic locations. Of the 348 *I. scapularis* (288 nymphal and 60 adult) ticks subjected to both nested PCR of the 16S-23S rRNA gene spacer and qPCR analysis, 78 (22.4%) ticks were positive and 239 (68.7%) ticks were negative by both methods, resulting in a concordance of 91.1% between nested and real-time qPCR. Of the remaining 31 ticks, 13 ticks were positive by nested PCR and 18 ticks were positive by qPCR. The overall positivity rates of ticks were 26.1% (91 of 348) by nested PCR and 27.6% (96 of 348) by qPCR ($P > 0.05$), indicating that the sensitivities of the two approaches were similar for detection of *B. burgdorferi* DNA in infected ticks.

TABLE 2. Genotype and spirochete loads in *I. scapularis* nymphs infected with *B. burgdorferi*^a

Genotype	No. of ticks infected (%)	Spirochete load in infected ticks ^b		
		Median	Mean	95% CI
RST1 only	19 (26)	1,675	1,980	890–4,393
RST2 only	15 (20)	3,775	4,224	1,715–10,374
RST3 only	29 (40)	2,550	2,212	1,113–4,384
Mixed infection	10 (14)	3,063	1,223	334–4,284
Total	73 (100)	3,175	2,264	1,512–3,385

^a Seventy-three (94%) of 78 PCR-positive ticks with RFLP data available were included.

^b The median numbers of spirochetes in infected ticks are based on original data from real-time qPCR. Means and 95% confidence intervals (95% CI) were back transformed.

DISCUSSION

In this study, the prevalence of infection and quantity of *B. burgdorferi* in 438 *I. scapularis* nymphal ticks and 60 adult ticks collected from various locations in the northeastern United States where Lyme disease is endemic were assessed using a real-time qPCR targeting the *B. burgdorferi*-specific *recA* gene. The sensitivity, specificity, and reproducibility of the qPCR targeting the *recA* gene have been assessed and reported previously (36). It is estimated that approximately 2,000 and 5,300 spirochetes were harbored in infected nymphal and adult *I. scapularis* ticks, respectively. Assuming that the efficiency of DNA extraction is not 100%, the actual number of spirochetes in infected ticks may be somewhat higher than those calculated in this study after measurement of *B. burgdorferi*-specific DNA by qPCR. Despite this, the spirochete density detected by qPCR in these field-collected *I. scapularis* ticks was comparable to those previously determined by microscopy-based assay and OspA antigen-capture ELISA (1, 3). In two early studies, approximately 2,000 spirochetes were reported to be harbored per nymphal tick collected from three sites in Massachusetts where Lyme disease is endemic (1), and 4,000 spirochetes were detected in adult ticks collected from Westchester County, New York (3). More recently, Rauter et al. reported that there were approximately 4,000 spirochetes per nymphal *I. ricinus* tick from southern Germany as determined by qPCR (30). Taken together, the data suggest that 2,000 to 4,000 spirochetes on average are present in the midguts of host-seeking nymphal ticks from a variety of areas where Lyme borreliosis is endemic.

In the present study, the sensitivity of the real-time qPCR protocol was comparable to that of a well-established nested PCR. However, the qPCR approach has certain advantages. First, the specificity of the qPCR product can be verified simply by analyzing the melting temperature after the PCR run. Additionally, real-time PCR can be performed in less than 1 h in a single capillary tube without opening the reaction tube, thereby significantly reducing the PCR running time and probability of cross-contamination. Thus, real-time qPCR analysis provides a rapid and reliable tool for the qualitative and quantitative analysis of *B. burgdorferi* sensu lato, and potentially other vector-borne pathogens, in field-collected ticks and should be useful for ecological and epidemiological surveillance of tick-borne diseases.

We have previously reported on the genotypic distribution of *B. burgdorferi* sensu stricto clinical isolates recovered from skin biopsy specimens of patients with early Lyme disease in Westchester County, New York, over a 7-year period (18). Of 183 skin isolates, 46 (25.1%) were type 1 (RST1), 70 (38.3%) were type 2 (RST2), and 55 (30.1%) were type 3 (RST3); the remaining 6.6% (12 of 183) were mixed cultures composed of at least two genotypically distinct isolates. RST2 isolates were cultured from skin biopsy specimens more frequently than either of the other two genotypes ($P = 0.07$ for comparison between type 1 and type 2; $P = 0.013$ for comparison between type 2 and type 3) (18). In this study, 26% (19 of 73) of the ticks were infected with RST1, 20.6% (15 of 73) were infected with RST2, 39.7% (29 of 73) were infected with RST3 and 13.7% were infected with a mixture of genotypes. The number of ticks infected by the RST3 genotype was higher than the number of

ticks infected with either the RST1 ($P = 0.07$) or RST2 ($P = 0.01$) genotype. Although these differences in the distribution of *B. burgdorferi* genotypes in isolates from ticks and patients may simply be due to sampling error, these results may also reflect differences in transmission of the distinct genotypes from ticks to humans. An additional explanation for the observed discrepancy may be differences in the pathogenicity of distinct genotypes of *B. burgdorferi*. This would be consistent with differences in pathogenicity observed for RST1 and RST3 isolates in a C3H/HeJ mouse model (37). As shown in this study, comparable numbers of spirochetes were detected in ticks infected with RST1 and RST3 genotypes of *B. burgdorferi* (with means of 1,980 and 2,212, respectively; $P > 0.05$). In contrast, the spirochete load, which correlates directly with the severity of disease, was at least twofold higher in the skin biopsy specimens of patients and in heart and joint tissues of mice infected with RST1 isolates than in those infected with RST3 isolates (19, 36, 37).

Although aggregation of macroparasites is well-known from the literature, microparasite aggregation has not been studied in great detail (20). Here we report a high degree of *B. burgdorferi* spirochete aggregation in host-seeking *I. scapularis* nymphs, with most infected ticks harboring few spirochetes ($<5,000$) and a small proportion of ticks with very large spirochete loads ($>20,000$) (Fig. 1A). Overdispersion is also the predominant pattern for macroparasites, including ticks, which are highly aggregated in nature (7, 29, 33). Our findings thus suggest a pattern of nested aggregation, with *B. burgdorferi* spirochetes aggregated in ticks and ticks aggregated in the field.

Aggregation of spirochetes in infected ticks may have important epidemiologic consequences. Heavily infected individuals in the tail end of aggregated distributions typically play a disproportionately large role in parasite transmission and natural maintenance (39). Similarly, the more heavily infected nymphs in the right tail of the frequency distribution (Fig. 1A) may play a more important role in maintaining *B. burgdorferi* in nature. Conversely, the nymphs with very few spirochetes may play little or no role in the natural maintenance of *B. burgdorferi*. Levin and Fish (16) found that 18.4% of *B. burgdorferi*-infected nymphs did not transmit spirochetes while feeding on white-footed mice (*Peromyscus leucopus*), which are natural reservoirs of infection. The number of organisms found in the 18% percent of infected ticks with the lowest number of spirochetes is ≤ 300 spirochetes (Fig. 1B), which may represent a transmission threshold, since ticks infected with few spirochetes cannot transmit infection (9). The mechanisms generating heterogeneous *B. burgdorferi* spirochete loads in ticks remain to be discovered.

We conclude that geographic and RST differences are not significant determinants of spirochete burden in ticks in northeastern United States. Pinchon et al. (26) found high heterogeneity in the number of *Plasmodium falciparum* gametocytes in the blood meal of mosquitoes feeding on the same host and an overdispersed distribution of gametocytes ingested by mosquitoes. Similarly, feeding behavior and spirochete maintenance may vary in ticks feeding on the same host in a way that generates an aggregated distribution of spirochete loads. Alternatively, the species and spirochete burden of the vertebrate host may influence the number of spirochetes acquired and

maintained by ticks. Further studies are needed to evaluate the influence of these possible mechanisms.

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