Transmission Dynamics of *Borrelia burgdorferi* s.s. During the Key Third Day of Feeding by Nymphal *Ixodes scapularis* (Acari: Ixodidae)

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ABSTRACT Nymphal *Ixodes scapularis* Say are the principal vectors of Lyme disease spirochetes (*Borrelia burgdorferi* sensu stricto) in the eastern United States. Physicians frequently face the decision of whether or not to administer prophylactic antibiotics to human tick bite victims in Lyme disease endemic regions, based on the overall probability that such bites will result in infection with *B. burgdorferi* s.s. We evaluated the transmission dynamics of *B. burgdorferi* s.s. during the key third day of nymphal *I. scapularis* feeding, when the risk of transmission rapidly increases. The cumulative probability that 50% of infected ticks transmitted *B. burgdorferi* s.s. occurred at 68 h of tick attachment and our overall estimate that a human tick bite would result in transmission of *B. burgdorferi* s.s. was 2.4%.

KEY WORDS ticks, Lyme disease, *Borrelia burgdorferi* s.s., *Ixodes scapularis*, transmission dynamics

In the northeastern United States, patients commonly detect and remove nymphal *Ixodes scapularis* Say during the act of tick feeding (Falco and Fish 1988, Falco et al. 1996, Rand et al. 2007). Because these ticks are widely known as the principal vectors of the Lyme disease spirochete *Borrelia burgdorferi* s.s. (Piesman 2002), patients who have removed ticks often consult medical or public health personnel after tick removal. Tick bite victims and their physicians must evaluate their risk of acquiring infection with the Lyme disease spirochete before deciding whether to embark on a prophylactic regimen of antibiotics (Nadelman et al. 2001). In general, physicians are advised to make the decision on whether or not to initiate prophylaxis for Lyme disease based on identification of the tick as a nymphal *I. scapularis*, the degree of endemicity for Lyme disease in the geographic vicinity of the tick bite, and the duration of tick attachment (Magid et al. 1992, Wormser et al. 2006).

A key factor in determining whether a tick has transmitted an infectious inoculum of Lyme disease spirochetes is clearly the duration of attachment. Using a murine model of infection, investigators repeatedly showed that the longer ticks were attached, the more likely they were to transmit infection with *B. burgdorferi* s.s. In North America, nymphal *I. scapularis* infected with *B. burgdorferi* s.s. were found to never transmit infection before 24 h of attachment and rarely transmit infection during the second day of tick feeding, whereas risk increased rapidly during the third day of tick feeding (Piesman et al. 1987, Piesman 1993a, des Vignes et al. 2001). Studies with patients in New York supported the paradigm developed in the murine model of increased transmission related to duration of attachment (Sood et al. 1997, Nadelman et al. 2001). In Europe, nymphal *I. ricinus* L. were capable of transmitting infection with *B. burgdorferi* s.l. (Kahl et al. 1998) or *B. afzelii* (Crippa et al. 2002) before 24 h of attachment, but risk increased with duration of attachment in this system also. Although the reasons for this delay in transmission of *B. burgdorferi* s.l. are not fully understood, it has been shown that spirochetes begin to decrease production of outer surface protein A (OspA) and increase production of OspC when tick feeding begins (Schwan and Piesman 2000, Ohnishi et al. 2001, Piesman et al. 2003). This in turn allows the spirochetes to be released from a tick midgut protein (TROSPA), migrate to the salivary glands, bind to a tick salivary gland protein (Salp15), and achieve transfer to the vertebrate host (Pal et al. 2004, Ramamoorthi et al. 2005, Rosa 2005, Hovius et al. 2007).

In this study, we sought to evaluate the transmission dynamics of *B. burgdorferi* s.s. during the key third day of nymphal *I. scapularis* feeding, when the risk of transmission rapidly increases. We determined the cumulative probability that nymphal *I. scapularis* will successfully transmit infection with Lyme disease spirochetes at specified times during attachment and compared that estimate to previously published studies.

Materials and Methods

Ticks and Mice. The *I. scapularis* tick colony used in this study was originally derived from ticks collected in Bridgeport, CT, in 2001. These ticks were main-

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tained and infected with the B31 strain of *B. burgdorferi* s.s. as previously described (Piesman 1993b). The mice used in this experiment were female Swiss Webster (4 wk old) from the pathogen-free colony maintained at the CDC Fort Collins laboratory. All animals in these experiments were handled according to approved protocols on file with the CDC/DVBID animal care and use committee. Individual nymphs were placed on individual mice held in cages over water. Mice were anesthetized, and ticks were allowed to attach over a period of 30 min. At specified intervals after attachment (48, 54, 63, and 72 h), mice were again anesthetized, and ticks were grasped with fine forceps and removed from the hosts. Ears, urinary bladder, and heart tissue were obtained from each mouse at 1 mo after tick removal. Tissues were processed and cultured in BSK media as previously described (Sinsky and Piesman 1989, Roehrig et al. 1992). Cultures were maintained at 33–34°C and examined at weekly intervals for 1 mo by darkfield microscopy for the presence of spirochetes. A total of 237 mice were exposed to individual nymphs; 159 mice had attached ticks on examination. The 159 individual nymphs were tested by polymerase chain reaction (PCR) to determine whether they were infected with *B. burgdorferi* s.s. Only those mice exposed to infected ticks were included in the analysis.

Nucleic Acid Assays. Feeding nymphal *I. scapularis* were removed from mice at specified time intervals between 48 and 72 h. Only one nymph was allowed to feed per animal. After the tick was removed, it was flash frozen in dry ice and ethanol and stored at −80°C. DNA was extracted from ticks by first using the 1.5-ml Fisherbrand disposable pestle system (Fisher, Pittsburgh, PA) with TRIzol reagent (Invitrogen, Carlsbad, CA) and then the DNA was isolated according to the manufacturer's instructions. DNA was resuspended in 200 μl 8 mM NaOH plus 20 μl 0.1 M HEPES. To calculate the numbers of *B. burgdorferi* s.s. in a sample, we performed quantitative PCR (qPCR) of the *flaB* locus, which is a single copy gene in the *B. burgdorferi* s.s. genome. The primers for *flaB* were *flaB*-F 5′-TCTTTTCTCTGGTGAGGGAGCT-3′ and *flaB*-R 5′-TCCTTCCTGTTGAACACCCTCT-3′. The qPCR analysis was performed in FastStart CYBR Green Master mix (Roche, Indianapolis, IN), with 300 nM of each primer and 2.5-μl sample (of the 220-μl DNA purification). Duplicate samples were analyzed in a 96 well-format with the following parameters; 1 cycle at 95°C for 10 min and 40 cycles at 95°C for 10 s, 60°C for 20 s, 72°C for 30 s, and recording at 74°C, using a Chromo4 real-time detection system (Bio-Rad Laboratories). At the end of each run, a melting curve analysis was performed to verify the correct product. Each 96-well plate was run with a 10-fold dilution standard curve of genomic DNA of *B. burgdorferi* s.s. (1.5×10^6–1.5×10^1 copies), which also served as a positive control; in addition, a water no-DNA control was performed on each plate. Crossing threshold (C_T), melting curves, and quantifications analysis were determined by the Chromo4 real-time detection system software. A PCR reaction was also included for each tick for the tick actin gene to be certain that no interference in the PCR reaction occurred from the presence of hemoglobin (data not shown).

Table 1. Point estimates of transmission of *B. burgdorferi* s.s. (B31) by individual colony-reared *I. scapularis* nymphs used in this study contrasted with previously reported estimates based on field and laboratory data (des Vignes et al. 2001).

Duration of attachment (h)	Hojgaard et al.	des Vignes et al.	Combined	Percent
24	ND	0/50[a]	0/50	0.0
48	0/31	4/50	4/81	4.9
54	3/30	ND	3/30	10.0
63	8/33	ND	8/33	24.2
72	23/29	36/52	59/81	72.8
96	ND	15/16	15/16	93.8

[a] No. of mice infected/no. mice exposed.
ND, not done.

Data Analysis. Using methods described previously (des Vignes et al. 2001), hourly estimates of the probability of transmission were modeled using the combined point estimates of transmission presented in Table 1. Briefly, we used a three-parameter Weibull distribution $T = \{1 - \exp[-\lambda^{\gamma}(t - G)^{\gamma}]\} \times k$, where T is the cumulative proportion of infected nymphal ticks transmitting *B. burgdorferi* s.s. by hour t. The minimum number of hours before transmission could occur is represented as G and is fixed at 24 h. The scale parameter $\lambda = 0.0219$ and shape parameter $\gamma = 6.89$ were estimated by minimizing the squared differences between observed and predicted values using JMP statistical software (SAS Institute, Cary, NC). Following des Vignes et al. (2001), a constant $k = 0.94$ was used to indicate that transmission levels off at 94% by 96 h. Feeding duration estimates (the amount of time ticks in the field actually fed on humans before detection and removal) were derived previously (Falco et al. 1996, des Vignes et al. 2001), and those estimates were used in this study to represent the probability of a tick detaching by hour t($f = \lambda e^{-\lambda t}$, where $\lambda = 0.0288$). The overall probability of an infected nymph transmitting *B. burgdorferi* s.s. was derived from $t = \sum_{t=24}^{96} f_t \times T_t$.

Results

A total of 159 nymphal *I. scapularis* fed on individual mice; of these, 123 ticks were judged to be infected with *B. burgdorferi* s.s. based on *flaB* qPCR. Individual ticks were allowed to feed on mice for 48, 54, 63, or 72 h. Of the 123 mice exposed, 34 (27.6%) became infected with *B. burgdorferi* s.s. (Table 1).

We used data obtained in this study and data generated in a previous study (des Vignes et al. 2001) to estimate the probability of ticks transmitting infection with *B. burgdorferi* s.s. as a function of time of attachment (Table 1; Fig. 1). The curve generated by the combined data set shows the probability of transmission to be slightly delayed compared with the previous estimate of des Vignes et al. (2001). The combined data set estimates that 50% of ticks transmit by 68 h of attachment, whereas the estimate of 50% transmission

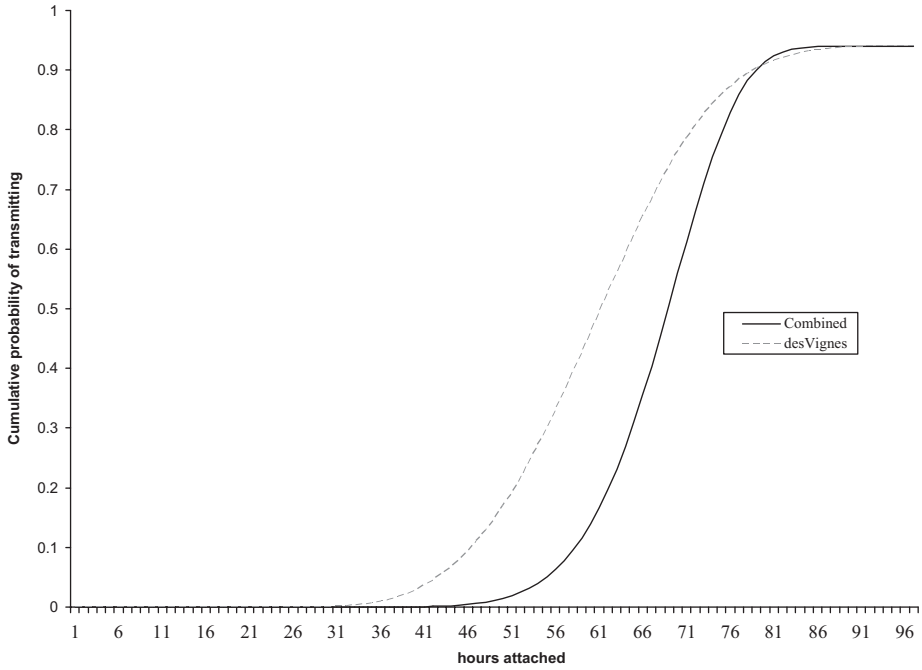

Fig. 1. Cumulative probability of infected *I. scapularis* nymphs transmitting *B. burgdorferi* s.s. by attachment time as estimated in a previous study (dashed-lines; des Vignes et al. 2001) and recalculated using data collected during this study combined with data from des Vignes et al. (2001).

was 60 h of attachment for the des Vignes et al. (2001)-generated curve. Most importantly, the combined data set using the equation $t = \Sigma_{t=24}^{96} f_t \times T_t$ produced an overall probability of 2.4% transmission for attached infected ticks.

Because *flaB* is a single copy gene, the locus can be used to estimate the overall load of spirochetes in a tick. We examined whether the level of *flaB* in each individual tick (spirochete equivalents) was related to the ability of that tick to transmit spirochetes (Table 2). The level of *flaB* was highest at 54 h of attachment in both ticks transmitting infection and ticks that failed to transmit. Although ticks that failed to transmit overall had higher levels of *flaB* compared with ticks that transmitted, there was no statistical difference between the two groups (Mann-Whitney *U* tests with χ^2 approximations; $P > 0.05$ for all comparisons) because of the enormous variation in *flaB* levels in individual ticks. We also attempted to examine the transcription levels of bacterial genes (*ospA* and *ospC*) and a tick salivary gland gene (*salp15*) that are important in transmission of *B. burgdorferi* s.s. but again saw tremendous variation from tick to tick (data not shown).

Table 2. Number of *flaB* copies (spirochete equivalents) in whole ticks removed from hosts at specified intervals

Duration of attachment (h)	Transmission status	
	Negative	Positive
48	146,256[a] (1,580–9,842,800)[b]	NA
54	625,548 (2,505–7,180,800)	607,772 (300,608–1,218,800)
63	584,188 (6,624–361,240)	363,418 (66,083–666,556)
72	139,326 (5,937–868,032)	209,968 (6,882–2,153,800)
Total sample	453,772 (1,580–9,842,800)	219,428 (6,882–2,153,800)

[a] Median value.
[b] Range of values.
NA, not applicable.

Discussion

Prospective studies of tick bite victims living in Lyme disease endemic regions showed that 1–3% of exposed individuals subsequently acquire infection with *B. burgdorferi* s.s. (Costello et al. 1989, Shapiro et al. 1992, Sood et al. 1997). A previous study suggested that the actual risk of transmission of *B. burgdorferi* s.s. from the bite of nymphal *I. scapularis* was 4.6% (des Vignes et al. 2001), which is higher than the range of the human studies cited above. The study by des Vignes et al. (2001) used field-collected nymphs and nymphs infected with the B31 or JD1 strain of *B. burgdorferi* s.s., whereas our study used nymphs infected solely with the B31 strain of *B. burgdorferi* s.s. In addition, the study by des Vignes et al. (2001) looked at transmission dynamics during 24-h intervals and did not closely examine key events during the third day of tick feeding. By combining the data obtained in this study with the data produced by des Vignes et al. (2001), we estimated that the probability of acquiring infection with *B. burgdorferi* s.s. from an infected nymphal *I. scapularis* bite was 2.4%, within the range of the human studies. Magid et al. (1992) suggested that all human tick bites by nymphal *I. scapularis* should be indicated to receive prophylactic

antibiotics if the overall risk was >3.5% but that prophylaxis should be preferentially considered on an individual basis if the risk was 1–3.5%; interestingly, our risk estimate falls within the preferential category, whereas the prior estimate of des Vignes et al. (2001) is in the indicated category.

Clearly, it would be useful to know the exact risk of a patient acquiring infection with *B. burgdorferi* s.s. based on an analysis of the individual tick rather than the overall risk in a given region. The decision as to whether to provide prophylactic treatment must be made quickly, because the recommended period for initiating prophylactic treatment is within 3 d of the tick bite (Nadelman et al. 2001, Wormser et al. 2006). In this study, we sought to determine whether the overall load of spirochetes present in an individual tick was associated with whether or not a nymphal *I. scapularis* transmitted infection with *B. burgdorferi* s.s. Unfortunately, the large variation from tick to tick was too great to produce an algorithm that would serve as useful guide for prophylaxis. Potentially, dissection of the tick's salivary glands and molecular analysis of the spirochetes and tick salivary gland proteins (Ohnishi et al. 2001, Piesman et al. 2001) could be more sensitive in predicting whether an individual tick will actually transmit infection. However, tick salivary gland dissection is not likely to become a widespread clinical tool with a rapid turn-around time. New quantitative tools, such as microarray analysis, may facilitate future attempts to develop algorithms for predicting if an individual tick will transmit pathogens. Meanwhile, the duration of tick attachment, as determined either from a scutal index (Falco et al. 1996) or careful patient exposure history, combined with prompt PCR testing of nymphal *I. scapularis* to determine whether the tick is infected with *B. burgdorferi* s.s., is still the best option for assisting clinicians considering antibiotic prophylactic treatment of tick bite victims. Wormser et al. (2006) recommended prophylaxis when the proportion of nymphs infected with *B. burgdorferi* s.s. exceeds 20% in a given region. Determining the duration of attachment and infection status of an individual attached tick may be a useful adjunct to this regional recommendation by Wormser et al. (2006).

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