Effect of Tick Removal on Transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* Nymphs

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The Journal of Infectious Diseases 2001; 183:773–8

The effect of feeding duration on pathogen transmission was studied for individual ticks infected with either laboratory or field strains of the Lyme disease spirochete *Borrelia burgdorferi* and field strains of *Ehrlichia phagocytophila*, an agent of human granulocytic ehrlichiosis. Infected nymphal *Ixodes scapularis* were allowed to feed individually on mice, and equal numbers were removed at 24-h intervals for ≤96 h. Mice were assayed for infection by culture, serologic testing, and polymerase chain reaction (PCR) analysis. Fed ticks were assayed by culture or PCR analysis. Transmission of *B. burgdorferi* did not occur during the first 24 h among 66 attempts, with maximum transmission occurring between 48 and 72 h. A model estimating the probability of infection from individual ticks removed by patients in a Lyme disease–endemic area yielded an overall probability of 4.6%. Infected *I. scapularis* nymphs transmitted *E. phagocytophila* within 24 h in 2 of 3 attempts, which indicates that daily tick removal may not be adequate to prevent human infection with this agent.

The principal vector of the Lyme disease spirochete, *Borrelia burgdorferi*, and of an agent of human granulocytic ehrlichiosis (HGE), *Ehrlichia phagocytophila*, in the eastern United States is the nymphal stage of *Ixodes scapularis* [1–4]. Infection prevalence of *B. burgdorferi* in *I. scapularis* nymphs in the northeastern United States is 15%–30% [3, 5, 6], and these ticks frequently feed on people living in Lyme disease–endemic areas [7–9]. However, Lyme disease is still a low-incidence disease, with rates of 67.9 per 100,000 in Connecticut, the state of highest risk [10]. Carefully controlled prospective human tick-bite studies have shown that only 1%–3% of bites by nymphal *I. scapularis* actually result in transmission of *B. burgdorferi* [11–13], despite a 10 times higher prevalence of infection in the biting tick population. The main reason why so few bites by infected ticks resulted in disease in these studies is that all ticks were removed from patients before completion of feeding, which prevented the transmission of spirochetes by most infected ticks. These studies demonstrate the value of daily tick inspections and prompt removal of attached ticks for preventing Lyme disease.

Laboratory studies have shown that nymphal *I. scapularis* do not transmit spirochetes during their first day of feeding, rarely do so during the second day of feeding, and efficiently do so only during and after the third day of feeding [14–16]. However, experiments on the transmission dynamics of *B. burgdorferi* have been conducted with groups of nymphs, thus requiring extrapolation to determine the efficiency of transmission by individual ticks. Also, these studies used only ticks infected in the laboratory with a single, cultured strain (JD1). Because of the genetic heterogeneity observed within *B. burgdorferi* populations in the field [17, 18], a more realistic appraisal of the effect of duration of tick attachment on transmission success should include field-collected nymphs. Accordingly, naturally infected nymphal *I. scapularis* were collected from 3 geographically separate areas where Lyme disease is endemic, and individual nymphs were allowed to feed on mice at durations of 1–4 days or to repletion. The efficiency of transmission for *B. burgdorferi* with these field-collected nymphs was compared with that of laboratory-reared ticks infected with cultured strains.

Both *B. burgdorferi* and *E. phagocytophila* can occur within a single population of ticks [19, 20]. For example, at a field site in Armonk, Westchester County, New York, 21% of field-collected *I. scapularis* nymphs tested positive for *E. phagocytophila*, and 26% were positive for *B. burgdorferi* by polymerase chain reaction (PCR) analysis. In addition, 6% of nymphs from the same population were coinfected with both pathogens [20]. Although nearly all human infections of *B. burgdorferi* are be-
Believed to be caused by nymphal stage I. scapularis ticks [21], a similar role for nymphs in the epidemiology of E. phagocytophila infection has not been determined. It is unknown how long I. scapularis nymphs naturally infected with E. phagocytophila must feed to infect a host. The outcome of interrupted feeding by infected nymphs is important to determine the risk to humans of contracting HGE, as well as Lyme disease, from individual tick bites. Thus, we measured the effect of feeding duration on the transmission of E. phagocytophila by individual field-collected I. scapularis nymphs and determined whether a single naturally coinfected nymph could simultaneously transmit E. phagocytophila and B. burgdorferi.

Materials and Methods

Experiments. In our first experiment, we examined the outcome of single ticks infected with laboratory or field strains of B. burgdorferi feeding on mice at 24-h intervals of removal. Transmission to mice was compared among strains for 3 removal intervals (24, 48, and 72 h) and until feeding to repletion (or ≤ 96 h, if not replete). The second experiment examined the same outcome for single ticks naturally infected with field strains of B. burgdorferi and E. phagocytophila. Transmission to mice was compared between these 2 pathogens at 3 removal intervals (24, 48, and 72 h).

Transmission of B. burgdorferi. Nymphal I. scapularis were collected from vegetation by use of drag cloths during the summers of 1995, 1996 and 1997. Ticks were collected from 3 areas where B. burgdorferi-infected ticks are known to be common: Lyme, Connecticut [6]; Naval Weapons Station Earle, Colts Neck, New Jersey [22]; and Armonk, New York [5]. Laboratory-reared nymphal I. scapularis infected with the B31 or JD1 strain of B. burgdorferi were produced as described elsewhere [23]. In brief, 4-week-old mice were fed on by 10–12 nymphal I. scapularis infected with either the B31 or JD1 strain of B. burgdorferi. One month after nymphal feeding, these mice were exposed to unfed larval I. scapularis. Larvae were allowed to molt to nymphs before use in experimental procedures. All ticks were held at saturated humidity, 21°C–22°C, before exposure to mice. Animals used in these experiments were 3–6-week-old males from a special pathogen-free colony of Imperial Cancer Research Fund outbred mice maintained at the Centers for Disease Control and Prevention Laboratory in Fort Collins, Colorado.

Individual nymphs were placed on mice and were allowed to feed ad libitum. At precisely 24, 48, or 72 h after attachment, mice were anesthetized with methoxyflurane (Mallinckrodt) and were searched for attached ticks. If no tick was found, the mouse was discarded from the experiment. When a tick was found, it was removed via gentle pressure with fine forceps and was assayed for infection.

Ticks tested for B. burgdorferi were surface-sterilized immediately after removal by immersion in 3% H2O2 for 3 min and in 70% ethanol for 3 min. Ticks then were homogenized in 0.3 mL of Barbour-Stoenner-Kelly (BSK) media (Sigma) and were cultured in 4-mL snap-cap tubes containing BSK, as described elsewhere [24]. Tick cultures were held at 33°C–34°C and were examined for viable spirochetes under dark-field microscopy on a weekly basis for 4 weeks. If tick cultures were negative at the end of 28 days, the mice they fed on were discarded. Mice that spirochete-positive ticks had fed on were held for 28 days and then were killed, at which time ear biopsy specimens and urinary bladder and heart tissue were obtained. Ear biopsy specimens were soaked for 15 min in disinfecting agent (Wescodyne; Amsco) and for 15 min in 70% ethanol. Urinary bladder and heart tissue were briefly soaked in 70% alcohol. Tissues were finely minced and were placed into 4-mL snap-cap tubes containing BSK. Cultures were held at 33°C–34°C and were examined weekly for spirochetes by dark-field microscopy. Feedings of individual ticks were conducted until ≥ 16 spirochete-positive ticks had fed on mice from each source (field, B31, and JD1) for each time interval (24, 48, and 72 h). In addition, 16 field-collected ticks were allowed to feed until repletion or for ≤ 96 h, if not replete.

Data analysis. To obtain hourly transmission estimates, we modeled B. burgdorferi transmission data for field-collected nymphs. For this, we used a 3-parameter Weibull distribution: 

\[ T = \left\{ 1 - \exp\left[ -\lambda \left( t - \gamma \right) \right] \right\} \times k, \]

where \( T \) is the cumulative proportion of infected nymphal ticks transmitting B. burgdorferi by hour \( t \) [25]. The parameter \( \gamma \), which represents the minimum number of hours before transmission could occur, was fixed at 24. The scale parameter \( \lambda = 0.0256 \) and shape parameter \( \gamma = 3.624 \) were estimated in Microsoft Excel 97 (Microsoft) by minimizing the squared differences between observed and model-predicted values. A constant, \( k = 0.94 \), was introduced on the assumption that transmission levels off to 94% at 96 h (table 1 and figure 1). Previously published estimates of the feeding duration of ticks found and removed by 444 humans in an area where Lyme disease is endemic.
[8] were modeled by using the exponential distribution $S = e^{-\lambda t}$, where $S$ is the proportion of ticks still attached by hour $t$ and parameter $\lambda = 0.0288$ (figure 2). The corresponding probability density function $f = \lambda e^{-\lambda t}$ provided proportion estimates of the ticks removed each hour. These hourly estimates were used to calculate the overall probability that an infected nymphal tick transmits $B. burgdorferi$ by summing, over each hour $t$, the probability of detaching multiplied by the probability of transmitting by that time: $t = \sum_{i=1}^{96} f \times T_i$.

**Dual infection experiment.** Ticks used in this second experiment were collected in Armonk (Westchester County, New York) in 1998. All 79 nymphs collected were individually fed on mice, as in the previous experiment, and were removed at 24 h intervals, as described above. Engorged nymphs were stored individually in 70% alcohol for later testing. Blood and serum samples were collected from mice before infestation, 12 days after infestation, and at death (28 days after infestation). Ear biopsy specimens and urinary bladder and heart tissue were obtained at death and were treated as described above for culture of $B. burgdorferi$.

PCR analysis was used to assay ticks and blood samples for the presence of $E. phagocytophila$ and ticks for the presence of $B. burgdorferi$. The positive control used for PCR analysis of $E. phagocytophila$ was an isolate from Westchester County (USG3) that is maintained in a promyelocytic leukemia cell line (HL-60; Aquila Biopharmaceuticals). The positive control used for PCR analysis of $B. burgdorferi$ was derived from purification of a positive cell culture of the B31 strain. The negative control used for both PCR assays was distilled water.

DNA was extracted from ticks and blood by use of a commercial DNA/RNA extraction kit (Isoquick; Orca Research), to maximize sensitivity [22]. For $E. phagocytophila$, primers EHR 521 (5'-TGT AGG CGG TTC GGT AAG TTA AAG-3') and EHR 747 (5'-GCA CTC ATC GTT TAC AGC GTG-3') were used to amplify a 247-bp fragment of 16S ribosomal DNA, as described elsewhere [26]. Primers FLA 297 (5'-CGG CAC ATA TTC AGA TGC AGA-3') and FLA 652 (5'-CCT GTT GAA CAC CCT CTT GAA CC-3'; provided by Erol Fikrig, Yale University) were used to amplify a 355-bp fragment of the flagellin gene of $B. burgdorferi$. The amplification products were electrophoresed in 2% agarose gels and were detected by staining with ethidium bromide and UV transillumination.

An indirect fluorescent-antibody test was performed on mouse serum; the test was developed by Aquila Biopharmaceuticals, using antigen derived from the same culture of $E. phagocytophila$ that were obtained from Westchester County [27]. Serum specimens were diluted 1:40 in PBS (pH 7.4) and were screened on spot slides of the *Ehrlichia* antigen.

**Results**

*Transmission of B. burgdorferi.* A pooled total of 413 field-collected ticks from New York, New Jersey, and Connecticut were exposed to mice, but 172 ticks did not attach to the hosts, and 13 nymphs produced contaminated cultures. Of the 228 remaining field-collected nymphs, 69 (30%) were infected with culturable $B. burgdorferi$. The mice exposed to these 69 nymphs were tested for infection. None of the 18 mice exposed to field-collected nymphs for 24 h acquired infection with $B. burgdorferi$, 2 (12.5%) of 16 exposed for 48 h became infected, 15 (78.9%) of 19 exposed for 72 h became infected, and 15 (93.8%) of 16 exposed for 96 h became infected (table 1). A model of these data shows that most transmission occurred 48–72 h after attachment (figure 1).

To estimate the predicted probability of $B. burgdorferi$ transmission resulting from a typical bite incident occurring in an area where Lyme disease is endemic, data on the probability of individual nymphs transmitting $B. burgdorferi$ over time (figure 1) were combined with published data on the duration of attachment of nymphs removed from 444 residents of Westchester County [8] (figure 2). The sum of the hourly products of the proportion of nymphs removed over time and the proportion of nymphs successfully transmitting over time produces an estimate of only 15.5% of infected nymphs transmitting *spirochetes* to humans who find and remove attached ticks. Considering that 30% of field-collected nymphs tested in this study were infected, we estimate that only 4.6% of all nymphal *I. scapularis* found and removed by patients in Lyme-disease endemic areas successfully transmit $B. burgdorferi$.

The efficiency of transmission for B31-infected nymphs was quite similar: 0% at 24 h, 11.8% at 48 h, and 70.6% at 72 h (table 1). Although nymphs infected with the JD1 strain of $B. burgdorferi$ seemed to be less efficient at transmitting an infectious dose of *spirochetes* (0% at 24 h, 0% at 48 h, and 56.2% at 72 h; table 1), the difference was not significant ($P = .07, \chi^2$ test).

**Dual infection.** In the second experiment, 79 field-collected nymphs were fed individually on mice; 39 nymphs fed for 24 h, 21 for 48 h, and 19 fed to near repletion in 72 h. Eight of the 39 ticks that fed for 24 h were positive for $B. burgdorferi$ by PCR analysis, but none of these mice became infected with $B. burgdorferi$. However, 3 of these nymphs tested positive for *E. phagocytophila*, and 2 were able to transmit infection to mice within 24 h, as indicated by both positive serologic testing and PCR analysis of blood.

For the 21 ticks removed at 48 h, 4 tested positive for $B.
burdorferi, and 1 tested positive for E. phagocytophila. Of the 4 mice fed on by nympha infected with B. burgdorferi, 2 became infected with B. burgdorferi, and the 1 mouse fed on by a nymph infected with E. phagocytophila became infected with E. phagocytophila.

Of the 19 ticks removed at 72 h, 4 tested positive for B. burgdorferi, and 4 tested positive for E. phagocytophila. Three of the 4 mice fed on by B. burgdorferi–infected ticks became infected with B. burgdorferi, and 2 of the 4 mice fed on by E. phagocytota–infected ticks became infected with E. phagocytophila. Furthermore, 1 tick at this interval was infected with both pathogens, and the mouse on which it fed became infected with both B. burgdorferi and E. phagocytophila. In all instances, mouse blood was positive by both serologic testing and PCR analysis.

Discussion

The relationship between the duration of attachment and the transmission efficiency of B. burgdorferi has been clearly established for spirochete strains of B. burgdorferi transmitted by I. ricinus in the northeastern United States. A similar relationship has been established for strains in the Pacific Northwest transmitted by Ixodes pacificus [28]. Several recent reports from Eurasia have suggested that Ixodes persulcatus ticks commonly transmit infection during the first day of attachment [29, 30]. In Europe, experimental transmission of B. burgdorferi sensu lato by Ixodes ricinus also was observed during the first day of attachment [31]. The relationship between the duration of attachment and the transmission efficiency should be studied experimentally with Borrelia afzelii, Borrelia garinii, and the Eurasian Lyme disease vectors I. ricinus and I. persulcatus. The possibility exists that partially fed infected ticks can reattach to a new host and rapidly transmit spirochetes during a second feeding [16]. The frequency with which this happens in nature is unknown, but it did not occur among the 66 field-collected ticks used in our experiments.

By combining data from our transmission experiment with data on feeding duration of ticks removed from humans [7], we estimated that only 4.6% of all nympha I. scapularis removed from tick-bite victims in areas where Lyme disease is endemic will successfully transmit B. burgdorferi. This estimate approaches the 1%-3% reported proportion of ticks transmitting infection in carefully controlled prospective tick-bite studies [11–13] and is slightly higher than the weighted rate of 1.4% resulting from a meta-analysis of 292 outcomes of untreated human tick bites [32]. However, our estimate of 4.6% was based on a 30% prevalence of B. burgdorferi in ticks. Geographic variation in B. burgdorferi infection prevalence in tick populations (15%–30%) would negate these small differences. Experimental and observed clinical rates of infection may also vary if there is any difference in susceptibility between humans and mice, the natural host for B. burgdorferi.

It is important to recognize that these low transmission estimates are only for ticks found and removed from patients. Infected ticks feeding to repletion in our experiment transmitted B. burgdorferi at a very high rate (93.8%), which indicates that transmission may approximate the infection prevalence found in nature (15%-30%) when ticks are not removed. Because most Lyme disease patients do not recall a tick bite [33], replete feedings are the obvious cause for most cases of Lyme disease.

Mice become infected with the agent of Lyme disease only after being exposed to an individual infected tick for ≥24 h. We did not attain transmission within 24 h in a total of 66 attempts in both experiments combined. By contrast, E. phagocytophila was transmitted to 2 mice within the first 24-h time interval. The reason for this difference in transmission rates can be attributed to the fact that B. burgdorferi resides in the gut of unfed ticks and must migrate to the salivary glands before transmission to the host can occur [34–36], whereas E. phagocytophila has been shown to reside in the salivary glands of I. scapularis ticks [37].

Our results differ from previously published results in that we obtained E. phagocytophila infection in mice within 24 h of tick attachment in 2 of 3 attempts. Katavolos et al. [38] reported a single incidence of infection within 24 h but did not observe other infections in any of 27 mice fed on by infected ticks for <36 h. Also, transmission of E. phagocytophila to mice only after ticks were attached for ≥48 h was reported by Hodzic et al. [39]. We have no explanation for these differing results, although our experiment used field-collected ticks, rather than laboratory ticks infected with a single strain.

Very few adult I. scapularis ticks transmit B. burgdorferi, because they are much larger in size than nympha and are more likely to be found and removed within 48 h of attachment [21]. Consequently, the time period for the risk of acquiring Lyme disease coincides primarily with seasonal activity of nympha ticks (June through August). However, if adult, as well as nymphal, I. scapularis transmit E. phagocytophila within 24 h of attachment, it would extend the risk for E. phagocytophila infection through the spring and fall months, when both adult and nympha ticks are active, and would increase the risk period for I. scapularis–borne pathogens by a factor of 3.

Of interest, 5 of 79 mice in our dual-infection study became infected with B. burgdorferi and 5 of 79 mice became infected with E. phagocytophila, despite the fact that the prevalence of infection with B. burgdorferi in the feeding ticks was twice that of E. phagocytophila (20% vs 10%). These results emphasize the potential difference in outcome of tick removal as a prophylactic measure for these tick-borne pathogens. B. burgdorferi transmission is time dependent, and removal of attached ticks before 48 h can be effective in preventing infection. However, transmission of E. phagocytophila in our experiment occurred within the first 24 h of tick attachment in 2 of 3 trials, which indicates that early tick removal may not be prophylactic against infection by this agent.

In one instance, a single nymph attached for 72 h transmitted
both *E. phagocytophila* and *B. burgdorferi* to a single mouse. Simultaneous infection in humans with both *E. phagocytophila* and *B. burgdorferi* has been reported [40]. Although it is possible that the 2 infections in this case were acquired independently from separate tick bites, our study confirms that both infections can be acquired from the bite of a single, naturally coinfected tick [41].

The results obtained in our study with individual field-collected nymphs reinforce the notion that daily inspections for vector ticks are an important and effective means of preventing Lyme disease. However, this method for disease prevention may have less significance for HGE and perhaps other tick-borne diseases.

**References**


