HHS Public Access

Author manuscript

Ticks Tick Borne Dis. Author manuscript; available in PMC 2021 January 01.

Published in final edited form as:

Ticks Tick Borne Dis. 2020 January ; 11(1): 101311. doi:10.1016/j.ttbdis.2019.101311.

Failure of the Asian longhorned tick, *Haemaphysalis longicornis*, to serve as an experimental vector of the Lyme disease spirochete, *Borrelia burgdorferi* sensu stricto

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Abstract

The invasive, human-biting Asian longhorned tick, *Haemaphysalis longicornis*, was detected in New Jersey in the eastern United States in August of 2017 and by November of 2018 this tick had been recorded from 45 counties across 9 states, primarily along the Eastern Seaboard. The establishment of *H. longicornis* in the United States has raised the questions of how commonly it will bite humans and which native pathogens may naturally infect this tick. There also is a need for experimental vector competence studies with native pathogens to determine if *H. longicornis* can acquire a given pathogen while feeding, pass it transstadially, and then transmit the pathogen in the next life stage. In this experimental study, we evaluated the vector competence of a population of *H. longicornis* originating from the United States (New York) for a native isolate (B31) of the Lyme disease spirochete, *Borrelia burgdorferi* sensu stricto (s.s.). In agreement with a previous experimental study on the vector competence of *H. longicornis* for *Borrelia garinii*, we found that uninfected *H. longicornis* larvae could acquire *B. burgdorferi* s.s. while feeding on infected *Mus musculus* mice (infection prevalence>50% in freshly fed larvae) but that the infection was lost during the molt to the nymphal stage. None of 520 tested molted nymphs were found to be infected, indicating that transstadial passage of *B. burgdorferi* s.s. is absent or rare in *H. longicornis*; and based on the potential error associated with the number of nymphs testing negative in this study, we estimate that the upper 95% limit for infection prevalence was 0.73%. An *Ixodes scapularis* process control showed both effective acquisition of *B. burgdorferi* s.s. from infected mice by uninfected larvae and transstadial passage to the nymphal stage (infection

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prevalence of 80–82% for both freshly fed larvae and molted nymphs). We also observed that although *H. longicornis* larvae could be compelled to feed on mice by placing the ticks within feeding capsules, attachment and feeding success was minimal (< 0.5%) when larvae were placed freely on the fur of the mice. We conclude that *H. longicornis* is unlikely to contribute more than minimally, if at all, to transmission of Lyme disease spirochetes in the United States.

Keywords

Borrelia burgdorferi sensu stricto; *Haemaphysalis longicornis*; *Ixodes scapularis*; Lyme disease; Transmission; Vector

1. Introduction

The Asian longhorned tick, *Haemaphysalis longicornis*, was unexpectedly detected in New Jersey in the eastern United States in August of 2017 (Rainey et al., 2018) and by November of 2018 the tick had been recorded from 45 counties across 9 states, primarily along the Eastern Seaboard (Beard et al., 2018). Environmental suitability models for *H. longicornis* in North America (Rochlin, 2019; Raghavan et al., 2019) suggest that this tick has the potential to establish across the eastern United States in the future. Moreover, the spread of *H. longicornis* in the United States is facilitated by its ability to parasitize both birds and large mammals, and that the invasive tick populations appear to be parthenogenetic (Hoogstraal et al., 1968; Heath, 2016; Beard et al., 2018; Tufts et al., 2019).

Haemaphysalis longicornis ticks are known to bite humans in Asia, Australia, and New Zealand (Hoogstraal et al., 1968; Lee et al., 2011; Heath, 2013; Barker and Walker, 2014; Yun et al., 2014), and a few human bites have now been recorded in the United States (Beard et al., 2018; Wormser et al., 2019). In its native range in Asia, *H. longicornis* is considered an important vector of human pathogens, most notably for severe fever with thrombocytopenia syndrome virus (Luo et al., 2015; Zhuang et al., 2018a). The establishment of *H. longicornis* in the United States has raised the questions of how commonly it will bite humans and which native pathogens may naturally infect this tick. Moreover, there is a need for experimental vector competence studies with key native pathogens to determine if *H. longicornis* can acquire a given pathogen while feeding, pass it transstadially, and then transmit the pathogen in the next life stage.

In Asia, *H. longicornis* ticks collected from the environment or from animals have been found naturally infected with various viral, bacterial, and parasitic pathogens affecting humans or domestic animals (Heath, 2002; Yu et al., 2016; Yun et al., 2016; Liu et al., 2017; Zhuang et al., 2018b), but experimental vector competence studies have focused primarily on *Babesia* and *Theileria* parasites (reviewed by Heath, 2002) and more recently viruses (Luo et al., 2015; Talactac et al., 2018; Zhuang et al., 2018a). In this study, we focused on evaluating the experimental vector competence of *H. longicornis* for the Lyme disease spirochete, *Borrelia burgdorferi* sensu stricto (s.s.), which is estimated to cause hundreds of thousands of human Lyme disease cases annually in the United States (Rosenberg et al., 2018). In Asia, host-seeking *H. longicornis* ticks have occasionally been found naturally infected with *Borrelia burgdorferi* sensu lato (s.l.) spirochetes, most commonly the human-

pathogenic *Borrelia garinii* (Chu et al., 2008; Meng et al., 2008; Sun et al., 2008; Murase et al., 2013; Yu et al., 2016). These findings are of concern because *H. longicornis* is now establishing in Lyme disease endemic areas of the northeastern United States, where it co-occurs with the Lyme disease spirochete vector, *Ixodes scapularis* (Beard et al., 2018; Eisen and Eisen, 2018; Tufts et al., 2019). However, previous experimental studies from Asia showed that while larval and nymphal *H. longicornis* and *Haemaphysalis concinna* ticks could acquire *B. garinii* with a blood meal from an infectious mouse host, the infection was uniformly lost during the molt to the subsequent life stage (Sun and Xu, 2003; Sun et al., 2003).

In this study, we examined if *H. longicornis* originating from the United States (New York) can serve as experimental vectors of a native isolate of *B. burgdorferi* s.s. (B31). This isolate originated from *I. scapularis* also collected in New York and was shown previously to be effectively acquired from infected mice by feeding *I. scapularis* larvae, passed transstadially and transmitted by the resulting nymphs during the subsequent blood meal (Des Vignes et al., 2001; Ohnishi et al., 2001; Piesman and Dolan, 2002; Jacobs et al., 2003; Hojgaard et al., 2008; Goddard et al., 2015).

2. Materials and methods

2.1. Sources of B. burgdorferi s.s.-infected I. scapularis nymphs used to infect experimental mouse hosts and larval H. longicornis and I. scapularis ticks

Pathogen-free *I. scapularis* larvae from the Medical Entomology Laboratory (MEL) colony at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, were infected with the *B. burgdorferi* s.s. B31 strain (isolated from *I. scapularis* ticks collected on Shelter Island, NY) via feeding on *Mus musculus* outbred CD1 mice (Charles River Laboratories, Wilmington, MA, USA) previously infected via tick bite. The resulting nymphs were then used to infect additional CD1 mice to serve as experimental sources of *B. burgdorferi* s.s. infection for uninfected *H. longicornis* and *I. scapularis* larvae originating from pathogen-free colonies maintained at the MEL. The *H. longicornis* larvae were F2 generation ticks from a colony established in 2018 using ticks collected in Westchester County, NY.

2.2. Challenge of experimental mouse hosts with B. burgdorferi s.s.-infected I. scapularis nymphs and confirmation of active infection in the mice

A total of 22 CD1 mice (1–3 mo old females; Charles River Laboratories) were each exposed to 10–15 potentially *B. burgdorferi* s.s.-infected *I. scapularis* nymphs placed freely on the mice and allowed to feed to repletion. The projected infection rate in these nymphs, based on testing of other unfed nymphs from the same source mice (as described in section 2.5), was 80–90%. Moreover, testing of the replete nymphs confirmed that each of the experimental mouse hosts was fed upon by ≥5 infected ticks (data not shown). To confirm active infection with *B. burgdorferi* s.s. in the mice, ear biopsies were taken 3 wk after the nymphal ticks were first introduced onto them (Sinsky and Piesman, 1989). The ear biopsies were surface sterilized in 70% ethanol for 5–10 min and then placed into in-house BSK-II culture medium with antibiotics (Cycloheximide, 20 μg/ml; Phosphomycin, 200 μg/ml; Rifampicin, 50 μg/ml; and Amphotericin B, 2.5μg/ml) at 34 °C. Culture aliquots were

examined under dark field microscopy at 400X magnification after 10 d, at which point the cultures from all 22 mice were spirochete-positive.

2.3. Attempted infestation of B. burgdorferi s.s.-infected mice with freely placed H. longicornis larvae

To determine the extent to which *H. longicornis* larvae will attach and feed to repletion when placed freely on the fur of experimental mouse hosts, we conducted an initial experiment where 5 *B. burgdorferi* s.s.-infected mice each were exposed to approximately 150 uninfected larvae introduced onto their backs toward the head and between the shoulder blades. The mice were anesthetized when the larvae were introduced onto them and we observed the larvae moving into the fur of the mice before the animals came out of the anesthesia. The mice were then housed in "tick drop-off" cages with a metal mesh flooring over a water surface to facilitate recovery of fed, detached ticks. By day 4 after the larvae were introduced, only 3 replete larvae had been recovered from the water and the mice did not harbor any additional larvae.

2.4. Infestation of B. burgdorferi s.s.-infected mice with uninfected H. longicornis and I. scapularis larvae confined to feeding capsules

To increase the feeding success of *H. longicornis* larvae on the infected mice, subsequent exposures were done with larval *H. longicornis* or *I. scapularis* ticks contained within feeding capsules attached to the shaved dorsal-midline of the mice, as described previously (Mbow et al., 1994; Soares et al., 2006). Infected mice were exposed to uninfected larvae 4–5 wk after they were infected via tick bite: 20 mice were infested with *H. longicornis* larvae (including the 5 mice described in section 2.3) and 2 process control mice were infested with *I. scapularis* larvae. To avoid crowding of feeding larvae within the single feeding capsule attached to each mouse, we introduced no more than 100 larvae per capsule. Because the mice may dislodge the feeding capsules over the duration of the tick blood meal, all mice were housed in "tick drop-off" cages with a metal mesh flooring over a water surface to facilitate recovery of fed, detached ticks that escaped the capsules. The feeding capsules and the water in the drop-off cages were inspected for replete larvae daily for 5 d after the larvae were introduced. A subset of the replete larvae were transferred to 70% ethanol, within 24 h of detachment, and the remainder were grouped by mouse host of origin into 5 ml capacity plastic vials with mesh lids (Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap, Thermo Fisher Scientific, Waltham, MA), which then were transferred to desiccators in a growth chamber (90–95% relative humidity; 22 °C and a 16:8 h light:dark cycle) while the larvae molted to nymphs. Subsets of the resulting nymphs were transferred to 70% ethanol either 1–2 wk or 5–6 wk after they molted. Replete larvae and molted nymphs were tested for presence of *B. burgdorferi* s.s. DNA as described in section 2.5 and nymphs were placed into culture to confirm the presence of viable spirochetes in the ticks, as described in section 2.6.

2.5. Detection of B. burgdorferi s.s. DNA from fed larvae and molted nymphs

Nucleic acids were isolated from fed larval or unfed nymphal ticks as follows. Individual ticks were homogenized in 350 μl of tissue lysis buffer (327.5μl ATL, 20 μl Proteinase K, 1 μg (1 μl) Carrier RNA, and 1.5 μl DX Reagent; Qiagen, Valencia, CA, USA) using a Mini-

Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) with 2.0 mm Very High Density Yttria stabilized zirconium oxide beads (GlenMills, Clifton, NJ, USA). Thereafter, DNA was extracted from the tick lysates using the KingFisher DNA extraction system (Thermo Fisher Scientific) and the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific) according to manufacturer recommendations. A blank was included as a negative control to ensure no cross-contamination occurred during the extraction.

The subsequent real-time TaqMan PCR reactions used an in-house multiplex master mix that included primers and probes (Table 1) for the following DNA targets: a pan-*Borrelia 16S rDNA* target (Kingry et al., 2018), the flagellar filament cap (*fliD*) target for *B. burgdorferi* s.s. (Hojgaard et al., 2014) and the *I. scapularis* actin target (Hojgaard et al., 2014) as the internal control. This tick actin target was found to amplify DNA also from *H. longicornis* and therefore could serve as a PCR and DNA purification control for both tick species. The multiplex PCR reactions were performed in 11 μl solutions with 5.5 μl iQ Multiplex Powermix (Bio-Rad, Hercules, CA, USA), 5 μl DNA tick extract, forward and reverse primers in a final concentration of 300 nM each, and probes in a final concentration of 200 nM each. Water was added to a final volume of 11 μl. The real-time TaqMan PCR cycling conditions consisted of: denature DNA at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 62 °C for 30 s on a CFX96™ Real-Time PCR Detection System (Bio-Rad). We analyzed samples using CFX Manager 3.1 software (Bio-Rad) with the quantitation cycle (Cq) determination mode set to regression. Based on Graham et al. (2018), only Cq values <40 were considered indicative of the pan-*Borrelia* and *B. burgdorferi* s.s. targets being present in the tested sample.

2.6. Confirmation of presence of viable B. burgdorferi s.s. spirochetes in molted nymphs

To confirm the presence of viable spirochetes in molted nymphs originating from mice that yielded nymphs testing positive for *B. burgdorferi* s.s. DNA by PCR, we placed groups of 2–4 ticks per mouse into culture. This included only *I. scapularis* nymphs, as no molted *H. longicornis* nymph tested positive for *B. burgdorferi* s.s. DNA by PCR (Table 2). Nymphs were surface sterilized in 70% ethanol for 5 min, sliced open with a scalpel to facilitate contact with the midgut material and then placed into in-house BSK II medium with antibiotics. Each of 5 culture tubes received 2–4 nymphs originating from larvae fed on the same mouse (3 culture tubes for mouse H65 and 2 for mouse H66). Cultures were examined for spirochetes as described in section 2.2.

2.7. Regulatory compliance

Animal use and experimental procedures were in accordance with an approved protocol on file with the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases Animal Care and Use Committee.

2.8. Statistical analysis

Based on the number of individual *H. longicornis* nymphs testing negative for infection with *B. burgdorferi* s.s. in the study, and using a uniform pool size of n =1, we used the Excel Add-In of Biggerstaff (2009) to calculate the maximum likelihood estimate and 95% confidence intervals for prevalence of infection.

3. Results

3.1. Outcome of feeds with H. longicornis larvae either placed freely or confined within feeding capsules on experimental mouse hosts

We first attempted to feed *H. longicornis* larvae on experimental mouse hosts infected with *B. burgdorferi* s.s. by placing ticks freely on the fur of 5 mice. Each of the mice received approximately 150 larvae, for an estimated total of roughly 750 larvae across the 5 mice. The larvae were observed to move into the fur when placed on the mice but over the subsequent days we collected numerous unfed larvae from the water in the tick drop-off cages the mice were housed in. In total, only 3 replete larvae were recovered from the mice, indicating that< 0.5% of the larvae placed freely on the mice attached and fed to repletion.

To improve the feeding success in subsequent work with experimental mouse hosts infected with *B. burgdorferi* s.s., we transitioned to introducing larval *H. longicornis* ticks into feeding capsules attached to shaved mouse skin. Of the 20 mice included in this experiment, 4 mice produced either no replete larvae (n=3 mice) or a single replete larva (n= 1 mouse); and we noted that 3 of these mice dislodged their feeding capsules within the first 48 h after larval introduction. As shown in Table 2, the remaining 16 mice yielded a total of 653 recovered replete larvae: mean=40.8 (±19.0) per mouse; median= 40.5 per mouse; range per mouse= 12–78. We also noted that no replete larvae were recovered in the first 72 h after introduction, whereas 121 (19%) were recovered at the 96 h time point and 532 (81%) at the 120 h time point. Of 621 replete larvae that were allowed the opportunity to molt to nymphs, a total of 556 (89.5%) molted successfully to active and vigorous nymphs within 3–4 wk.

3.2. Acquisition of B. burgdorferi s.s. by uninfected H. longicornis or I. scapularis larvae fed upon experimental mouse hosts with culture-confirmed active infections, and transstadial passage to the resulting nymphal life stage

As shown in Table 2, we recovered > 10 replete larvae from 18 mice with culture-confirmed active *B. burgdorferi* s.s. infections, including 16 mice exposed to uninfected *H. longicornis* larvae and 2 process control mice exposed to *I. scapularis* larvae. Testing of replete larvae, collected within 24h of detachment from the mice, revealed the presence of *B. burgdorferi* s.s. DNA in 80% of 10 tested *I. scapularis* larvae and 56% of 32 tested *H. longicornis* larvae (Table 2). However, we note that Cq values for the *B. burgdorferi* s.s. *fliD* target tended to be higher (suggestive of lower numbers of spirochetes) for the 18 positive *H. longicornis* larvae (range of 31.1–38.4; median value=34.8) compared to the 8 positive *I. scapularis* larvae (range of 26.2–29.9; median value=27.2). The prevalence of infection remained high (82%) for 40 *I. scapularis* nymphs tested for presence of *B. burgdorferi* s.s. DNA 1–2 wk after their molt (range of Cq values for positive nymphs of 31.6–37.6; median value of 32.8); and we further documented viable spirochetes via culture from 5 different groups of nymphs (Table 2). In striking contrast, none of 520 molted *H. longicornis* nymphs contained detectable *B. burgdorferi* s.s. DNA; this included 292 nymphs harvested 1–2 wk after the molt and 228 nymphs harvested 5–6 wk after the molt (Table 2). Based on the potential error associated with the sample size in our study, with all 520 nymphs testing negative, we estimate that the upper 95% limit for infection prevalence was 0.73%. Overall, both *I. scapularis* and *H.*

longicornis larvae were capable of acquiring spirochetes while feeding on infectious mice but only *I. scapularis* was found to pass spirochetes transstadially to the nymphal life stage.

4. Discussion

Our finding that *H. longicornis* larvae could acquire *B. burgdorferi* s.s. while feeding on infected mouse hosts but that the infection was lost during the molt to the nymphal stage indicates that this tick is unlikely to contribute more than minimally, if at all, to transmission of Lyme disease spirochetes in the United States. This is in agreement with previous studies from China showing that *H. longicornis* and *H. concinna* could acquire another human-pathogenic *B. burgdorferi* s.l. spirochete, *B. garinii*, while feeding on infectious mouse hosts in the larval or nymphal stage but that infection was uniformly lost during the molt to the subsequent nymphal or adult life stage (Sun and Xu, 2003; Sun et al., 2003). For both *B. burgdorferi* s.s. and *B. garinii*, spirochete DNA was detected in > 50% of *H. longicornis* larvae 1 d after they completed the blood meal but was absent from molted nymphs (Table 2; Sun et al., 2003). Moreover, in the case of *H. longicornis* and *B. garinii*, spirochete DNA was detected in replete larvae up to 8 d after they completed the blood meal but absent thereafter, and culture yielded live spirochetes only during the first 1–2 d after the larvae completed the blood meal (Sun et al., 2003). It also should be noted that we tested two groups of molted nymphs, harvested at 1–2 wk and 5–6 wk after the molt (Table 2), to rule out the possibility that spirochete numbers in the ticks were below our level of detection shortly after the molt but thereafter increased over time in the molted nymphs to reach detectable levels. Due to the lack of molted *H. longicornis* nymphs infected with *B. burgdorferi* s.s., we were not able to assess if infected *H. longicornis* nymphs may be capable of transmitting this spirochete.

Similar to our results for *H. longicornis*, transstadial passage of *B. burgdorferi* s.s. from infected fed larvae to molted nymphs was absent or very rare in experimental studies on other human-biting non-*Ixodes* ticks in the United States, including *Amblyomma americanum*, *Dermacentor variabilis*, *Dermacentor andersoni,* and *Dermacentor occidentalis* (Piesman and Sinsky, 1988; Mather and Mather, 1990; Brown and Lane, 1992; Mukolwe et al., 1992; Ryder et al., 1992; Oliver et al., 1993; Lane et al., 1994; Sanders and Oliver, 1995; Dolan et al., 1997; Piesman and Happ, 1997; Eisen and Lane, 2002). The failure of *Dermacentor* ticks to serve as vectors of *B. burgdorferi* s.s. has been attributed to the presence of specific tick antimicrobial peptides (defensins) that are lytic to the spirochetes (Johns et al., 2000, 2001a, 2001b; Sonenshine et al., 2002, 2005; Chrudimska et al., 2014). Intriguingly, a similar phenomenon was recently reported in an in-vitro assay where exposure to functional segments of *H. longicornis* defensins HlDFS1 and HlDFS2 were shown to negatively impact the *B. burgdorferi* s.s. 297-GFP strain (Sun et al., 2017). Another important question is to what extent *H. longicornis* immatures will feed on key natural reservoirs for *B. burgdorferi* s.s. in the United States, such as the white-footed mouse (*Peromyscus leucopus*), *Tamias* spp. chipmunks, and *Blarina* spp. and *Sorex* spp. shrews (LoGiudice et al., 2003; Hanincova et al., 2006; Brisson et al., 2008). In New Zealand and in its native range in Asia, *H. longicornis* immatures have occasionally been recorded from rodents (Hoogstraal et al., 1968; Tenquist and Charleston, 2001) but data on how commonly rodents are infested are scarce. One notable study from China recorded a total of < 10

immature *H. longicornis* ticks recovered from 257 examined rodents collected during the active period for *H. longicornis* larvae and nymphs in an area where host-seeking ticks were abundant: all ticks were recovered from the greater long-tailed hamster (*Cricetulus triton*) whereas no *H. longicornis* ticks were found on *Apodemus* spp. or *M. musculus* mice (Zheng et al., 2012). Another recent study from China failed to recover *H. longicornis* immatures from 1781 rodents collected in an area where domestic animals and hares (*Lepus sinensis*) were infested with this tick (Zheng et al., 2019). In areas of the northeastern United States where it is now establishing, *H. longicornis* co-occurs with *I. scapularis* (Beard et al., 2018; Tufts et al., 2019) and therefore has distinct potential to encounter *B. burgdorferi* s.s.-infected hosts. However, a recent study on Staten Island, NY, recorded no *H. longicornis* immatures on rodents (*P. leucopus* and *Tamias striatus*) or shrews (*Blarina brevicauda*) in areas where host-seeking immature ticks were present and white-tailed deer (*Odocoileus virginianus*) were infested with all life stages of this tick (Tufts et al., 2019). This field observation agrees with our finding in the laboratory that *H. longicornis* larvae introduced freely onto the fur of *M. musculus* mice appear unwilling to attach and feed on these rodents. Additional field studies targeting rodents and shrews in areas where host-seeking *H. longicornis* immatures are abundant are needed to clarify the likelihood of this tick to become involved in natural transmission of the human pathogens these small mammals carry, including causative agents of Lyme disease (*B. burgdorferi* s.s. and *Borrelia mayonii*), anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia microti*) and Powassan virus disease.

Acknowledgments

This research was supported through a fellowship with the Oak Ridge Institute for Science and Education (ORISE) administered by Oak Ridge Associated Universities (ORAU), and funded by the Centers for Disease Control and Prevention.

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Table 1

Primers and probes used for PCR targets in the in-house multiplex master mix.

Target	Primer/probe	Sequence (5'– 3')[a]	Reference
B. burgdorferi s.s. fliD	fliD-F	TGGTGACAGAGTGTATGATAATGGAA	Hojgaard et al., 2014
	fliD-R	ACTCCTCCGGAAGCCACAA	
	fliD-probe	FAM-TGCTAAAATGCTAGGAGATTGTCTGTCGCC-BHQ1	
Borrelia 16S rDNA	16S rDNA-F	AGCYTTTAAAGCTTCGCTTGTAG	Kingry et al., 2018
	16S rDNA-R	GCCTCCCGTAGGAGTCTGG	
	16S rDNA-probe	HEX-CCGGCCTGAGAGGGTGAWCGG-BHQ1	
I. scapularis actin	actin-F	GCCCTGGACTCCGAGCAG	Hojgaard et al., 2014
	actin-R	CCGTCGGGAAGCTCGTAGG	
	actin-probe	Q670-CCACCGCCGCCTCCTCTTCTTCC-BHQ3	

[a]BHQ1, BHQ3: Black Hole Quencher 1 and 3, respectively; FAM: 6-carboxyfluorescein; HEX: hexachlorofluorescein phosphramidite; Q670: Quasar 670.

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Table 2

Acquisition of *B. burgdorferi* s.s. from infected *M. musculus* mice by uninfected *H. longicornis* or *I. scapularis* larvae and transstadial passage of spirochetes to the nymphal life stage.

Mouse ID	Infection status of mouse[b]	No. fed larvae recovered	Detection of *B. burgdorferi* s.s. DNA in fed larvae harvested within 1 d of detaching			Detection of *B. burgdorferi* s.s. DNA in nymphs harvested 1–2 wk after molting			Detection of *B. burgdorferi* s.s. DNA in nymphs harvested 5–6 wk after molting			Confirmation of viable spirochetes in molted nymphs[c]
			No. tested larvae	No. positive larvae	% infected larvae	No. tested nymphs	No. positive nymphs	% infected nymphs	No. tested nymphs	No. positive nymphs	% infected nymphs	
H. longicornis												
H46	+	50	2	1	50	20	0	0	27	0	0	–
H48	+	21	2	0	0	17	0	0	–	–	–	–
H49	+	48	2	2	100	20	0	0	15	0	0	–
H50	+	13	2	2	100	9	0	0	–	–	–	–
H51	+	42	2	1	50	20	0	0	19	0	0	–
H52	+	34	2	0	0	20	0	0	10	0	0	–
H53	+	56	2	2	100	20	0	0	28	0	0	–
H55	+	73	2	2	100	20	0	0	30	0	0	–
H56	+	12	2	1	50	9	0	0	–	–	–	–
H57	+	30	2	1	50	20	0	0	7	0	0	–
H58	+	78	2	2	100	20	0	0	29	0	0	–
H59	+	24	2	2	100	17	0	0	–	–	–	–
H60	+	39	2	0	0	20	0	0	8	0	0	–
H61	+	52	2	2	100	20	0	0	26	0	0	–
H62	+	34	2	0	0	20	0	0	11	0	0	–
H64	+	47	2	0	0	20	0	0	18	0	0	–
All mice		653	32	18	56	292	0	0	228	0	0	
I. scapularis												
H65[a]	+	72	5	3	60	20	13	65	–	–	–	+
H66[a]	+	36	5	5	100	20	20	100	–	–	–	+
All mice		108	10	8	80	40	33	82				

[a] Process control mouse exposed to *I. scapularis* larvae.

[b] +, *B. burgdorferi* s.s. detected by culture of spirochetes from ear biopsies.

[c] +, *B. burgdorferi* s.s. detected by culture of spirochetes from ticks.