

Vector Competence of *Ixodes scapularis* and *Ixodes ricinus* (Acari: Ixodidae) for Three Genospecies of *Borrelia burgdorferi*

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ABSTRACT The vector competence of 2 tick species, *Ixodes ricinus* (L.) and *Ixodes scapularis* Say, was determined and compared for 3 genospecies of *Borrelia burgdorferi*. The 3 genospecies of *B. burgdorferi* used in the following experiments were *Borrelia burgdorferi* sensu stricto (B-31 and B-31.D1 clone), *Borrelia afzelii* (strain Pgau.C3), and *Borrelia garinii* (strain VS286 and VSBP). Spirochetes from all 5 strains were inoculated intradermally into outbred mice; larval ticks of both species were subsequently fed on those mice and replete larvae were assayed for infection by culture in BSK-H media every 7 d for 4 wk. Infection frequencies in *I. scapularis* exposed to the 5 strains were as follows: B-31 (90%), B-31.D1 (83%), Pgau.C3 (87%), VS286 (10%), and VSBP (5%). The comparable infection frequencies for *I. ricinus* were B-31 (3%), B-31.D1 (3%), Pgau.C3 (90%), VS286 (5%), and VSBP (3%). Resultant nymphal *I. scapularis* successfully transmitted B-31, B-31.D1, Pgau.C3, and VS286 to outbred mice. *I. ricinus* nymphs transmitted Pgau.C3 and VS286. Both species failed to transmit strain VSBP.

KEY WORDS *Ixodes ricinus*, *Ixodes scapularis*, *Borrelia burgdorferi*, vector competence

LYME DISEASE is the most common human vector-borne disease in the United States and Europe (Strle and Stantic-Pavlinic 1996, Dennis 1995). The Lyme disease spirochete *Borrelia burgdorferi* sensu lato is maintained in zoonotic cycles involving primarily small rodent hosts and is transmitted by ticks of the genus *Ixodes* (Lane et al. 1991). The primary vector of Lyme disease in North America is *Ixodes scapularis* Say; *Ixodes ricinus* (L.) serves as the primary vector in Europe (Burgdorfer et al. 1983). The principal vector of the Lyme disease spirochete in Asia is *Ixodes persulcatus* (Schulze) (Takada et al. 1994). Laboratory colonies of *I. scapularis* infected with *B. burgdorferi* have been produced in natural zoonotic cycles between tick and rodent (Piesman 1993). The standard procedure for maintaining high prevalence of infection in these colonies involves exposing rodents to infected nymphs, and subsequently feeding colony larvae on these naturally infected rodents. These practices routinely result in the production of large numbers of infected nymphal *I. scapularis*. In contrast, infection of laboratory *I. ricinus* with *B. burgdorferi* has been achieved mainly through artificial means involving

capillary feeding of spirochetes in BSK-H medium, and exposing mice to these infected nymphs (Hu et al. 1992; Gern et al. 1993, 1994; Kurtenbach et al. 1994).

A great deal of genetic diversity exists within *B. burgdorferi* sensu lato (Postic et al. 1994, Fukunaga et al. 1996, Mathiesen et al. 1997). Only 3 genospecies, however, are associated with human Lyme disease: *B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii* (Baranton et al. 1992, Canica et al. 1993, Anthonissen et al. 1994). These 3 genospecies exist concomitantly in endemic areas of Europe (Nohlmans et al. 1995, Péter et al. 1995, Strle et al. 1995). The search for a Lyme disease vaccine has focused primarily on the outer surface protein (OspA) as the test antigen (Fikrig et al. 1990, Gern et al. 1994, Golde et al. 1995). Unfortunately, the 3 disease-causing genospecies of *B. burgdorferi* exhibit variation in their OspA proteins (Péter and Bretz 1992, Marconi et al. 1993). A vaccine consisting of the OspA protein from 1 genospecies may not protect against all 3 genospecies (Schaible et al. 1993, Lovrich et al. 1993). Therefore, a multivalent OspA vaccine may be essential in Europe to protect against Lyme disease (Gern et al. 1997).

OspA vaccines appear to work by killing spirochetes in the vector tick before these spirochetes have a chance to migrate from the tick midgut and switch the predominant Osp from OspA to OspC (Fikrig et al. 1992, Schwan et al. 1995, de Silva et al. 1996). Thus, evaluation of vaccine efficacy should be assessed using tick transmission (Fikrig et al. 1995). Moreover, ticks infected with each of the 3 genospecies would allow for a heterologous tick challenge to assess comprehensively protection afforded by candidate antigens.

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Accordingly, as a prelude to multivalent vaccine trials, we infected laboratory colonies of *I. scapularis* and *I. ricinus*, with all 3 genospecies of *B. burgdorferi*.

Materials and Methods

Tick Colonies. Larval *I. scapularis* used in these experiments came from adult ticks flagged in Westchester County, NY, in 1994 (WC '94); this colony was highly susceptible to *B. burgdorferi* and free of transovarially acquired spirochetes (Piesman et al. 1996). Colony *I. ricinus* were derived from nymphal and adult ticks flagged in Neuchâtel, Switzerland, during the first 2 wk of June 1994. These *I. ricinus* ticks were shipped to the Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Infectious Diseases, Fort Collins, CO, for the purpose of establishing a laboratory colony. All ticks were held at 21°C, 97% RH, and a photoperiod of 16:8 (L:D) h. Twenty-five field-collected adult female *I. ricinus* (along with 20 males) fed on a rabbit. A total of 3 adult females oviposited and derived larvae was included in the colony. Approximately 1/4 of each resultant F_1 larval batch was surface sterilized and cultured in BSK-H media as unfed larvae; all these F_1 larvae were negative on culture. An ear biopsy was performed on the rabbit exposed to the adult ticks; this culture was negative. In addition, 275 field-collected *I. ricinus* nymphs were fed on a total of 8 ICR outbred mice from a specific pathogen-free colony maintained at the Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, CO; 255 replete nymphs were collected. All 8 of these mice were ear biopsy culture positive (Sinsky and Piesman 1989); the mice were determined to be infected with *B. afzelii* by pulsed-field gel electrophoresis (PFGE) of all isolates (data not shown). Replete nymphs were allowed to molt to adults and fed on the ears of a rabbit. Skin from the rabbit's ear was cultured and proved negative for spirochetes. Twenty-five adult females derived from these field-collected nymphs were fed on a rabbit. Twelve fed to repletion and oviposited, and their resultant F_1 larvae were added to the colony. Again, $\approx 1/4$ of flat F_1 larvae were cultured in BSK-H media. None of these larvae produced spirochetes. Approximately 3,000 unfed F_1 larval *I. ricinus* were cultured in the course of establishing this colony; none produced spirochetes on culture.

Spirochete Strains. Three genospecies of *B. burgdorferi* consisting of 5 different isolates were used as follows: VS286 (*B. garinii* tick isolate [Péter et al. 1995]), VSBP (*B. garinii* human isolate [provided by O. Péter]), Pgau.C3 (*B. afzelii*; a cloned derivative of Pgau [Wilske et al. 1988]), B-31 (passage 6, provided by Alan Barbour, University of California, Irvine; strain originally isolated from Shelter Island, NY [Burgdorfer et al. 1982]), and B-31.D1 (B-31 clone). The European strains were both characterized and typed by using the monoclonal antibody D6 and genetic analysis as previously described (Péter et al. 1995, Péter et al. 1998).

Mouse Inoculations. Groups of 3 ICR outbred mice were inoculated with each of the 5 *Borrelia* isolates.

Borrelia were grown in BSK-H media and $\approx 1 \times 10^5$ spirochetes were inoculated intradermally into each mouse. Mice were assayed for infection at 21 d after inoculation by ear biopsy (Sinsky and Piesman 1989); 14 of 15 mice were ear biopsy culture positive. The 1 negative mouse (exposed to B-31) was excluded from the study. Animals were handled according to procedures outlined in a protocol on file with the animal care and use committee at the Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, CO.

Tick Infestations and Culture. Approximately 250 uninfected larval *I. ricinus* and *I. scapularis* were placed separately on ear biopsy positive mice, day 28 after inoculation, from each of the 5 groups and allowed to feed to repletion. Ten replete larvae of each tick species that had fed on mice infected with each of the 5 strains were surface sterilized and cultured in BSK-H media. Surface sterilization consisted of a 3-min soak in hydrogen peroxide, followed by a 10-min soak in 70% EtOH. Ticks were then transferred to 4-ml glass tissue grinders and triturated in 0.5 ml of BSK-H culture medium (Pollack et al. 1993) containing 6% rabbit serum and antibiotics (rifampin, 50 $\mu\text{g}/\text{ml}$; cycloheximide, 200 $\mu\text{g}/\text{ml}$; phosphomycin, 200 $\mu\text{g}/\text{ml}$; and fungizone, 2.5 $\mu\text{g}/\text{ml}$). The mixture was decanted into 4-ml tubes containing BSK-H medium and incubated at 34°C. These procedures were performed at zero, 7, 14, 21, and 28 d after repletion. Cultures were examined for spirochetes as previously described (Dolan et al. 1997).

Upon molting (44–60 d after repletion), 20 resultant nymphs of each tick species from each infected mouse were cultured to assay for transstadial infection. Cultures were read by dark-field microscopy every 7 d for 4 wk. Remaining nymphs were both individually fed and group fed on clean ICR outbred mice, to test for spirochete transmission to a subsequent host. Although *I. scapularis* nymphs were placed directly on mice, *I. ricinus* nymphs were placed in a feeding capsule attached to the mouse as previously described (Mbow et al. 1994). Capsules were required for *I. ricinus* nymphal feeding, because these ticks attach to mice less readily than do *I. scapularis*. Mice were assayed for infection 28 d after nymphal drop-off by ear biopsy (Sinsky and Piesman 1989). Again cultures were read every 7 d for 4 wk.

Xenodiagnosis of Tick-Infected Mice. In an additional attempt to produce *B. garinii*-infected nymphs, 1 of the mice that tested ear biopsy positive after being fed on by *B. garinii*-infected *I. ricinus* nymphs, received a subsequent infestation of *I. ricinus* larvae. These mice were exposed to larvae at 5 wk after exposure to nymphs. Resultant, engorged larvae were assayed for infection by culture at 2 wk after feeding; cultures were examined weekly for spirochetes by dark-field microscopy for 4 wk.

Results

Ixodes ricinus. Larval *I. ricinus* demonstrated the ability to acquire all 3 genospecies of *Borrelia* (Table

Table 1. Ability of larval *I. ricinus* and *I. scapularis* to acquire and maintain 5 strains of *B. burgdorferi* (*B. b.*)

Tick species and strain of <i>B. b.</i>	No. infected/no. examined					Total (%)
	Days after repletion					
	7	14	21	28	≥44 ^a	
<i>I. ricinus</i>						
<i>B. afzelii</i>	8/10	9/10	10/10	9/10	18/20	54/60 (90)
<i>B. garinii</i> (VS286)	0/10	1/10	0/10	2/10	0/20	3/60 (5)
<i>B. garinii</i> (VSBP)	1/10	1/10	0/10	0/10	0/20	2/60 (3)
B-31 (passage 6)	1/10	1/10	0/10	0/10	0/20	2/60 (3)
B-31.D1 (clone)	0/10	2/10	0/10	0/10	0/20	2/60 (3)
<i>I. scapularis</i>						
<i>B. afzelii</i>	10/10	8/10	9/10	8/10	17/20	52/60 (87)
<i>B. garinii</i> (VS286)	1/10	1/10	2/10	2/10	0/20	6/60 (10)
<i>B. garinii</i> (VSBP)	1/10	0/10	2/10	0/10	0/20	3/60 (5)
B-31 (passage 6)	9/10	8/10	9/10	10/10	18/20	54/60 (90)
B-31.D1 (clone)	8/10	8/10	7/10	9/10	18/20	50/60 (83)

^a After molting to nymphs (44–60 d after repletion).

1). Only *B. afzelii*, however, demonstrated efficient transstadial survival to resultant nymphs. Overall infection frequencies of *I. ricinus* for *B. burgdorferi* sensu stricto (B-31 low), *B. burgdorferi* sensu stricto (B-31.D1 clone), *B. afzelii* (Pgau.C3), *B. garinii* (VS286), and *B. garinii* (VSBP) were 3, 3, 90, 5, and 3%, respectively.

Resultant nymphs from 3 of the 5 groups were allowed to feed on naive ICR outbred mice. A range of 10–30 nymphal ticks was fed on each mouse. Resultant nymphs that had fed on mice infected with *B. burgdorferi* sensu stricto were not fed on naive ICR outbred mice because of low infection rates and the small numbers of these ticks that were available. Nymphal ticks were able to transmit 1 of 2 *B. garinii* isolates (VS286) and *B. afzelii* (Pgau.C3) (Table 2). Nymphal *I. ricinus* did not successfully transmit VSBP (*B. garinii*), an isolate originally made from human CSF.

One of the ICR outbred mice that had been naturally infected by being fed on by *B. garinii* (VS286)-infected *I. ricinus* nymphs was exposed to uninfected *I. ricinus* larvae. Larvae were allowed to feed ad libi-

tum, collected, and reassayed for infection by culture; 6 of 20 replete larvae (30%) cultured were positive. A 10-fold increase resulted from feeding larvae on mice infected with VS286 by tick-bite compared with larvae feeding on needle-inoculated animals.

Ixodes scapularis. Larval *I. scapularis* were fed on mice infected with *B. burgdorferi* sensu stricto (B-31 low), *B. burgdorferi* sensu stricto (B-31.D1), *B. afzelii* (Pgau.C3), *B. garinii* (VS286), and *B. garinii* (VSBP). Replete larvae were cultured as mentioned above. Overall infection frequencies for the 5 aforementioned isolates were 90, 83, 87, 10, and 5%, respectively. Resultant nymphs were fed on naive ICR outbred mice. *I. scapularis* was vector competent for 4 of the 5 spirochete isolates. The success of transmission to a subsequent host (Table 3) was 100% for *B. burgdorferi* sensu stricto (B-31 low), *B. burgdorferi* sensu stricto (B-31.D1 clone), *B. afzelii* (Pgau.C3), and 25% for *B. garinii* (VS286). *I. scapularis* was unable to transmit the human isolate (VSBP) of *B. garinii* to naive ICR outbred mice.

Discussion

In the current study we successfully infected *I. ricinus* and *I. scapularis* with all 3 genospecies of *B.*

Table 2. Ability of *I. ricinus* nymphs to transmit 3 strains of *B. burgdorferi* (*B. b.*)

Trial	Strain of <i>B. b.</i>	No. of nymphs fed on mouse	Ear biopsy result
1	<i>B. afzelii</i>	14	+
2	<i>B. afzelii</i>	25	+
3	<i>B. afzelii</i>	17	+
4	<i>B. garinii</i> (VS286)	9	—
5	<i>B. garinii</i> (VS286)	23	—
6	<i>B. garinii</i> (VS286)	18	+
7	<i>B. garinii</i> (VS286)	24	—
8	<i>B. garinii</i> (VS286)	26	—
9	<i>B. garinii</i> (VS286)	20	+
10	<i>B. garinii</i> (VS286)	21	—
11	<i>B. garinii</i> (VS286)	19	—
12	<i>B. garinii</i> (VS286)	25	+
13	<i>B. garinii</i> (VSBP)	26	—
14	<i>B. garinii</i> (VSBP)	27	—
15	<i>B. garinii</i> (VSBP)	21	—

Transmission experiments were not conducted with B-31 and B-31.D1 due to small numbers of infected nymphs available.

Table 3. Ability of *I. scapularis* nymphs to transmit 5 strains of *B. burgdorferi* (*B. b.*)

Trial	Strain of <i>B. b.</i>	No. of nymphs fed on mouse	Ear biopsy result
1	<i>B. afzelii</i>	7	+
2	<i>B. afzelii</i>	16	+
3	<i>B. garinii</i> (VS286)	14	+
4	<i>B. garinii</i> (VS286)	12	—
5	<i>B. garinii</i> (VS286)	24	—
6	<i>B. garinii</i> (VS286)	21	—
7	<i>B. garinii</i> (VSBP)	15	—
8	<i>B. garinii</i> (VSBP)	11	—
9	<i>B. garinii</i> (VSBP)	23	—
10	<i>B. garinii</i> (VSBP)	21	—
11	B-31 (passage 6)	10	+
12	B-31 (passage 6)	6	+
13	B-31.D1 (clone)	8	+
14	B-31.D1 (clone)	7	+

burgdorferi. This was accomplished by the natural route of feeding larvae on infected mice. In contrast, most experiments employing infected *I. ricinus* nymphs used ticks infected by artificial means. Gern et al. (1994), Hu et al. (1992), and Kurtenbach et al. (1994) reported using *I. ricinus* nymphs infected by capillary feeding BSK-H medium containing spirochetes. Although effective, this method seems to be more time-consuming and less efficient than acquiring spirochetes naturally by feeding on an infected host. Moreover, dispersal of spirochetes within artificially infected ticks may differ from that within naturally infected ones. Allowing ticks to become infected by feeding on mice also mimics what occurs in nature. Mice infected by tick-bite are more infectious than needle-inoculated mice. Gern et al. (1993) reported that mice infected by the natural route with infected *I. ricinus* ticks were substantially more infective for ticks by 2- to 6-fold than mice experimentally inoculated. Piesman (1993) reported similar findings in *I. scapularis*. Our work determined that rodents infected via tick feeding or inoculated with tick homogenates were more infectious to ticks than rodents infected with cultured spirochetes. In the current study a 10-fold increase occurred in the infection frequency of larval *I. ricinus* when they were allowed to feed on naturally infected mice as compared with needle-infected mice.

OspA vaccines act by preventing spirochetes from leaving the tick midgut and migrating to the salivary glands prior to transmission to the host (Fikrig et al. 1992, de Silva et al. 1996), suggesting that a valid test of a vaccine candidate antigen requires a natural tick challenge. In addition, the observation that a given OspA antigen derived from 1 genospecies may not protect against all 3 disease-causing genospecies of *B. burgdorferi* (Schaible et al. 1993, Lovrich et al. 1993) requires that ticks infected with each genospecies be available to test a multivalent vaccine. Most vaccine trials have used ticks infected in the laboratory with only *B. burgdorferi* sensu stricto (Gern et al. 1994, Golde et al. 1995). One vaccine trial used a combination of ticks collected in the field in the northeast United States, California, Sweden, and Germany, as well as ticks exposed to characterized strains (Fikrig et al. 1995). Fikrig et al. (1995) showed that a single antigen produced from the OspA gene derived from *B. burgdorferi* sensu stricto could afford protection against both *B. burgdorferi* sensu stricto and *B. afzelii*. These researchers failed, however, to infect rodents with *B. garinii*-infected ticks. The current study should facilitate efforts to produce *B. garinii*-infected ticks, enabling vaccine candidates to be tested against ticks infected with each of the 3 disease-producing genospecies of *B. burgdorferi* (Gern et al. 1997).

The principal reservoir hosts for *B. burgdorferi* sensu stricto and *B. afzelii* are clearly rodents. In the northeastern United States, the white-footed mouse, *Peromyscus leucopus*, is the primary reservoir of *B. burgdorferi* sensu stricto (Mather et al. 1989); in the Rocky Mountains, the Mexican woodrat, *Neotoma mexicana*, is the primary reservoir (Maupin et al.

1994), and the dusky-footed woodrat, *Neotoma fuscipes*, and the California kangaroo rat, *Dipodomys heermanni*, serve as primary reservoirs in the Pacific Northwest (Brown and Lane 1992). In Europe (Humair et al. 1995) and in Japan (Nakao and Miyamoto 1995) *Borrelia* isolates from rodents appear to be principally *B. afzelii*. Humair et al. (1995) reported a specific association exists between *B. afzelii* and rodent reservoirs in Europe.

Our research shows the *I. ricinus* used in these experiments to be most susceptible to *B. afzelii*. *I. ricinus* collected in the field used to start the laboratory colony were fed on New Zealand white rabbits and ICR outbred mice. Cultured ear biopsies from the mice yielded 8 *B. afzelii* isolates. According to Hu et al. (1992), *B. burgdorferi* sensu stricto is the predominant genospecies in the area where the *I. ricinus* ticks were collected, followed by *B. afzelii*. Strle et al. (1995) reported isolating *B. afzelii* only from rodents in 3 of 6 sites studied in Slovenia. Moreover, Humair et al. (1995) reported isolating only 1 genospecies, *B. afzelii*, from rodent ears in 2 Lyme disease-endemic areas of Switzerland, despite isolating all 3 genospecies from the ticks in the same 2 areas. Their findings of heterogeneity of tick isolates and homogeneity of rodent isolates suggests that an affinity exists between *B. afzelii* and rodents, and indicates other genospecies may be associated with other vertebrate hosts.

Several studies have demonstrated that both *I. ricinus* and *I. scapularis* frequently parasitize and acquire *Borrelia* infections from birds (Magnarelli et al. 1992, Battaly and Fish 1993, Humair et al. 1993, Isogai et al. 1994, Stafford et al. 1995, Piesman et al. 1996). Mehl and Traavik (1983) demonstrated *I. uriae* associated with seabird colonies. Olsen et al. (1995) found seabirds infected with *Borrelia*. Sequence analysis revealed DNA obtained was from *B. garinii*. The relatively low body temperature of 38°C in certain seabirds compared with the body temperature of terrestrial birds (40°C) suggests seabirds may be competent hosts for *Borrelia* spirochetes (Olsen et al. 1995, Piesman et al. 1996). These findings suggest birds may play an important role as reservoirs for *Borrelia*, particularly *B. garinii*. That *B. garinii* is associated with nonrodent hosts may explain the difficulty of getting *B. garinii* into a mouse model. Our research, however, shows that it is possible to infect *I. ricinus* and *I. scapularis* with *B. garinii* by feeding larvae on naturally infected mice.

Assessment of protection in mice, using the natural vector, requires infected ticks. One could consider using field-collected ticks; however, the risk involved is the potential transmission of other pathogens. Ticks are excellent vectors of disease-producing agents and can be simultaneously infected with pathogens other than *B. burgdorferi* such as *Babesia microti*, human granulocytic ehrlichiosis, and tick-borne encephalitis. Laboratory-reared colonies can be generated and maintained free of pathogens other than *B. burgdorferi* (Piesman et al. 1986, Telford et al. 1996, Benda 1958). In addition, vaccines that induce anti-tick immunity may act against a broad array of tick-borne pathogens

simultaneously (Wikel et al. 1997). Therefore, the need for infected ticks to test the efficacy of anti-tick vaccines and vaccines against various etiologic agents is essential.

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