APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1998, p. 3089–3091 Vol. 64, No. 8
0099-2240/98/$04.00+0

Failure of *Ixodes* Ticks To Inherit *Borrelia afzelii* Infection

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Received 13 March 1998/Accepted 4 May 1998

To define conditions promoting inherited infection by Lyme disease spirochetes in *Ixodes* ticks, we variously infected ticks with *Borrelia afzelii* and examined their progenies by dark-field microscopy, immunofluorescence, PCR, and serial passage. No episode of inherited infection was evident, regardless of instar or gender infected or frequency of exposure. We suggest that these spirochetes rarely, if ever, are inherited by vector ticks.

Although about a quarter of nymphs and somewhat more adults may naturally be infected by Lyme disease spirochetes, *Borrelia burgdorferi* sensu lato, such pathogens infect relatively few larvae, generally less than 1% of those sampled from the field (4, 7, 10, 12, 14, 15, 19, 23). Prevalence greatly exceeds these estimates in one European sample (21) and slightly exceeds them in several others (5, 9, 24). The scarcity of spirochetal infection in larval ticks, which generally is taken as evidence of transovarial transmission, suggests that vector ticks may only infrequently inherit infection (14, 19). Inherited infection generally, but not always (17, 22), appears to be more frequent in studies of larvae that are reared from field-derived adult females. Lyme disease spirochetes have been detected in all progeny of 5% of infected adults (2), half of the eggs of infected adults (16), and all first-generation as well as second-generation progeny of an infected tick (11). The contradiction inherent in these estimates of the frequency of transovarial transmission requires analysis based on defined conditions. Larval *Ixodes* ticks may most frequently inherit infection by Lyme disease spirochetes if their parents had been exposed to infection repeatedly or during some particular developmental stage. Accordingly, we compared the frequencies of infection by a field-derived isolate of Lyme disease spirochetes in larval *Ixodes ricinus* ticks reared from parents that had fed on infectious hosts during a particular developmental stage or sequence of stages.

I. ricinus ticks, isolated in Berlin, Germany, in 1994, were reared in the laboratory with Mongolian jirds, *Meriones unguiculatus*, as hosts for the subadult stages and rabbits, *Oryctolagus cuniculus*, for the adults. These ticks were in their third generation of continuous laboratory rearing and had never been exposed to infected hosts; batches are routinely screened for infection.

To obtain a well-characterized spirochete isolate directly from the field, nymphal ticks collected in Berlin, Germany, in 1996 were permitted to feed individually on jirds. Two weeks later, laboratory-reared, spirochete-free larvae were permitted to engorge on each of these jirds. One aliquot of each cohort of the resulting nymphs was examined for spirochetal infection by dark-field microscopy. Spirochetal DNA in the midguts of a second aliquot of the infected cohorts was amplified by nested genospecific PCR that amplified a fragment of the gene encoding the outer surface protein A. The sensitivity of an amplification protocol (3) was enhanced by adding a pair of outer primers: (5′-3′) GGTCTAATATTAGCCTTAATAGCATG and GAGTCGTATTTTTGTACTGTTATTGTGT. Buffers and thermocycling conditions (6) were modified by adding 2.5 mM $MgCl_2$ and by subjecting samples to 40 cycles of 30 s of denaturation at 96°C and the first annealing reaction for 30 s at 52°C with a 45-s extension at 72°C and the second annealing reaction for 30 s at 58°C with a 30-s extension at 72°C. The last extension at 72°C lasted 2 min. Subsequently, 10 μl of the product was amplified with the inner pair of primers. The resulting information served as the basis for selecting a *Borrelia afzelii*-infected jird for use in the experiments that followed.

Ticks that had engorged on the designated *B. afzelii*-infected jird were used to infect the jirds or rabbits that ultimately were used to infect the experimental ticks. Larvae from one clutch of eggs were divided into cohorts, and each was permitted to feed on infected or noninfected hosts as required. Subadult ticks were brushed onto jirds individually caged over water which was examined twice daily; larvae and nymphs were removed promptly. Adults, feeding on the ears of rabbits, were confined in cloth bags. Detached ticks were removed twice daily. At about 4 weeks after they hatched, larvae were examined for inherited spirochetal infection by PCR (6), dark-field microscopy, and direct immunofluorescence.

Experiments were designed to determine whether inherited *B. afzelii* infection depends upon the frequency of feeding of ticks on infected hosts. Subadult ticks were permitted variously to feed on jirds and adults were permitted to feed on rabbits to include all seven possible combinations of infected and noninfected hosts. Ticks fed on infected hosts as often as three times, once during each of their trophic developmental stages (Table 1). Males sampled from each cohort were examined to determine whether the cohort had acquired infection during a prior developmental stage. Virtually all (26 of 29) were infected. Adult females from each cohort were permitted to engorge and to oviposit.

Spirochetal infection in F1 progeny was detected directly, either by microscopy or by PCR. About 10 larvae from each female were examined for spirochetal infection by dark-field microscopy, and about 100 larvae from each female were examined in pools of 50 by PCR. In addition, we attempted to detect spirochetes by immunofluorescence in about 10 larvae from each of four selected egg batches. In all, we analyzed 4,932 larvae derived from 48 adult females. No spirochetal infection was detected. We conclude that few, if any, progeny

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TABLE 1. Absence of *B. afzelii* spirochetes in larval *I. ricinus* ticks sampled from individual progenies deriving from ticks that had engorged in their subadult stages on infected jirds or as adults on infected rabbits

Developmental stage infected			No. of progenies examined	No. of larvae examined by:	
Larva	Nymph	Female		Microscopy	PCR
+	−	−	13	112	1,140
+	+	−	12	141	1,100
+	−	+	7	64	700
+	+	+	2	24	200
−	+	−	11	120	1,000
−	+	+	1	10	100
−	−	+	2	21	200
			48 (total)	492 (total)	4,440 (total)

of infected female ticks inherit spirochetal infection, regardless of the parental instar that was infected or their frequency of exposure to infected hosts.

To enhance sensitivity of spirochete detection, infection in F1 progeny was detected indirectly by serial passage. Batches of 100 larvae sampled from the progenies of seven spirochete-infected females were permitted to feed on individual spirochete-free jirds. Their mothers had acquired infection either during their larval stage, their larval and nymphal stages, or their nymphal stage or as female adults. Two weeks later, spirochete-free xenodiagnostic larvae were permitted to feed on each of these jirds, and the resulting nymphs were examined by dark-field microscopy. No spirochetes were detected in these ticks. These xenodiagnostic observations confirmed that larvae do not frequently inherit spirochetal infection from their mothers.

Finally, we determined whether spirochetes infecting male ticks may be transmitted to their progeny venereally, via the spermatophore. Accordingly, 13 spirochete-free, virgin female ticks were confined with infected males for a week, and each pair was permitted subsequently to engorge on a spirochete-free rabbit. Four of these males had been infected as larvae, four had been infected as nymphs, and five had been infected both in their larval and in their nymphal stages. Spirochetal infection in each of these males was verified by dark-field microscopy after its mate had engorged. Each such female produced a batch of fertile eggs, thereby demonstrating that a spermatophore had been received from its infected mate. Of the larvae that resulted, 1,296 were examined for spirochetal infection by dark-field microscopy and PCR. None was infected. Spirochetal infection in a male tick, therefore, appears not to be inherited by its progeny.

Lyme disease spirochetes disseminate from their midgut developmental site more rapidly than do those tick-borne pathogens that are destined mainly to be inherited. Such spirochetes traverse the hemolymph of adult ticks before they become replete (20) and invade the salivary glands (25) when these glands are enlarging and synthesizing saliva most rapidly. Vertically transmitted pathogens, in contrast, disseminate from the gut postprandially, reaching their target tissue long after the adult tick has detached and when vitellogenesis is proceeding rapidly (8). Tick-borne pathogens generally invade the ovaries or salivary glands of the vector when these tissues metabolize most actively. To the extent that Lyme disease spirochetes are adapted to a salivarian route of transmission, coordination with vitellogenesis would be precluded.

Experimental analysis of the conditions influencing transovarial transmission of Lyme disease spirochetes may require many observations because these spirochetes rarely infect more than 1% of larval vector ticks in nature (14). Our sample of 100 eggs from each of 48 egg clutches, however, provides reasonable confidence that we have excluded such a possibility, both within and between clutches. In addition, spirochetes infected virtually all of the adult ticks that produced these eggs; less than half of naturally infected females contain Lyme disease spirochetes. The sensitivity of our experimental design for detecting such rare events, therefore, seems adequate due to the numerous ticks that were examined.

By varying the developmental stage of the tick that was exposed to spirochetal infection, we sought to explore all possible schedules of spirochetal invasion of the ovary. Opportunity for ovarian penetration was increased by superinfecting ticks that had imbibed spirochetes in a previous instar. No particular schedule of infection appears to be associated with transovarial transmission.

Field-derived larval ticks may falsely appear to have inherited infection, if they had engorged partially on a spirochete-infected host and had detached prematurely. Indeed, larvae detach prematurely from resting white-footed mice in nature (19a). Such questing larvae may previously have become infected, while transiently attached to an infected host (18). Those that feed on certain hosts, such as voles, are particularly prone to detach after taking only a small quantity of food (13), and these animals may have predominated on the grass-covered island from which an intensely infected lot of larvae recently was sampled (21). It may be that larval ticks in nature become infected mainly by sipping from an infected host rather than by inheritance.

We considered the possibility that venereally transmitted spirochetal infection may be inherited more readily than is food-borne infection. *Ixodes persulcatus* ticks are said to transmit Lyme disease spirochetes venereally because ticks maintained in pairs were twice as likely to be infected as were those held alone (1). No offspring, however, were examined for spirochetal infection. Even if spirochetes were to be transmitted from an infected male via its spermatophore to its previously spirochete-free mate, our observations indicate that few, if any, offspring of such infected male ticks would acquire infection.

The peculiar requirement of Lyme disease spirochetes to disseminate from the gut of the tick before it detaches from its host suggests that these spirochetes may be specialized for penetrating salivary glands rather than ovarian tissue. Previously reported natural spirochetal infection in larval ticks may reflect interrupted feeding. We suggest that vector ticks rarely, if ever, inherit infection by the Lyme disease spirochete.

(This research was conducted in partial fulfillment of the doctoral degree from the Humboldt-Universität zu Berlin [T. W. Schinkel] and from the Freie Universität Berlin [B. Klug].)

This study was supported by grant Ma 942/7-1 from the Deutsche Forschungsgemeinschaft.

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