Frequency and efficiency of transovarial and subsequent transstadial transmissions of Borrelia burgdorferi sensu lato in Ixodes ricinus Ticks

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FREQUENCY AND EFFICIENCY OF TRANSOVARIAL AND SUBSEQUENT TRANSSTADIAL TRANSMISSIONS OF *BORRELIA BURGDORFERI* SENSU LATO IN *IXODES RICINUS* TICKS

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Abstract

We investigated the frequency and efficiency of transovarial transmission of *Borreliae* in *Ixodes (I.) ricinus,* the European tick vector of *Borrelia burgdorferi* sensu lato. Field collected females were allowed to feed on rabbits. *Borreliae* were detected by direct immunofluorescence in the midgut of 65/447 (14.5%) females after oviposition, and 21/65 (32.3%) were systemically infected with spirochetes in their ovary. Transovarial transmission rate by field collected females systemically infected was investigated and only 3/21 (14.3%) females with *Borrelia* in their ovaries transovarially transmitted borreliae to their filial generation. To study transovarial and subsequent transstadial maintenance of spirochetes in F1 and F2 generations, eggs (n=17620) or larvae (n=3490) of a batch of 358 females were examined for spirochetes. The infection rates in the egg or larva batches varied from 44% to 100%. Spirochetes were observed in 85.8% and 83% of F1 larvae and nymphs, respectively. Eleven percent (17/116) of F1 female ticks examined after oviposition were found infected and 82.4% (14/17) had spirochetes in their ovary. Ovary infection were significantly more present in F1 females (82.4%) than in females collected from nature (32.3%, p=0.0003) and similarly F1 females (76.9%) with ovary infection more frequently transmitted spirochetes to their progeny than field-collected females with ovary infection (14.3%, p=0.0007). F1 and F2 immature ticks transmitted spirochetes to mice.

Résumé

Dans ce travail nous avons étudié la fréquence et l'efficacité de la transmission transovarienne de borrélies par la tique *Ixodes (I.) ricinus,* le vecteur européen de *Borrelia burgdorferi* sensu lato, l'agent de la borréliose de Lyme. Des tiques femelles ont été récoltées dans la nature puis nourries sur des lapins. Des borrélies ont été observées par immunofluorescence (IF) dans l'intestin de 65/447 (14.5%) femelles examinées après la ponte. Parmi les 65 femelles infectées, 21 (32.3%) présentaient une infection systémique avec des

spirochètes dans les ovaires. Seules 3/21 (14.3%) femelles présentant des borrélies dans leurs ovaires ont transmis l'infection à leur descendance. Afin d'étudier la transmission transovarienne et le maintien transstadial de l'infection au cours des générations, nous avons examiné la descendance de 358 femelles. Un total de 17620 oeufs et 3490 larves ont été examinés. Leurs taux d'infection variaient entre 44% et 100%. Des spirochètes ont été observés chez 85.8% et 83% des larves et nymphes, de la première génération. Onze pourcent (17/116) des femelles de première génération examinées après la ponte étaient infectées et 82.4% (14/17) présentaient une infection des ovaires. L'infection des ovaires était significativement plus souvent présente chez les femelles de première génération (82.4%) que chez les femelles récoltées dans la nature (32.3%, p=0.0003). De même, les femelles de première génération présentant une infection ovarienne ont transmis plus fréquemment l'infection à leur descendance (76.9%) que les femelles récoltées dans la nature présentant une infection ovarienne (14.3%, p=0.0007). Les borrélies transmises par voie transovarienne et par voie transstadiale au cours des générations sont restées infectieuses pour la souris puisque les descendants de la première et de la deuxième génération ont transmis les borrélies aux souris.

INTRODUCTION

Ixodes (I.) ricinus is the main vector of *Borrelia burgdorferi* sensu lato (sl), the causative agent of Lyme borreliosis, in Europe. In unfed *I. ricinus*, *B. burgdorferi* sensu lato (sl) is mainly found in the tick midgut (Burgdorfer *et al.*, 1983, Gern *et al.*, 1990). However, systemic infections in unfed and feeding ticks with *Borrelia* infecting additional organs, including ovarial tissues have been reported (Gern *et al.*, 1990, Lebet & Gern 1994, Leuba-Garcia *et al.*, 1994, Gern *et al.*, 1996). The presence of *B. burgdorferi* in tick ovary can result in the transmission of spirochetes from *I. ricinus* females to their progeny (Burgdorfer *et al.*, 1983, 1989, Stanek *et al.*, 1986, Monin *et al.*, 1989).

Although it is known that spirochetal infection in larvae can be inherited from *I. ricinus* females, the frequency of the occurrence of transovarial transmission among a large population of females remains unknown. In nature, the prevalence of *B. burgdorferi* sl infection in host-seeking *I. ricinus* larvae averages 1.9% (Hubalek *et al.*, 1998) which indicates that either the frequency or the efficiency of transovarial transmission is low.

The present study was initiated to determine the frequency and efficiency of transovarial transmission of *B. burgdorferi* for maintenance of borrelia infection in *I. ricinus* until the second generation (F2). The ability of transovarially infected larvae (F1 and F2) and nymphs (F1) to transmit spirochetes to mice was also investigated.

MATERIALS AND METHODS

Ticks: *I. ricinus* adults were collected by flagging vegetation in two forested areas highly endemic for *B. burgdorferi* sl located on the Swiss Plateau (Switzerland): Neuchâtel (Canton Neuchâtel) and Staatswald (Canton Bern) from 1991 to 1995. Males and females were placed on the ear of New Zealand white rabbits and engorged females were maintained individually in tubes until egg laying. Larvae and nymphs were fed on Swiss mice. Ticks were kept at room temperature and 98% relative humidity as described by Graf (1978).

Detection of B. burgdorferi *in ticks:*

A direct immunofluorescence assay (IFA) was used to detect spirochetes in *I. ricinus* as described previously (Gern *et al.*, 1991). Briefly, eggs, larvae, nymphs and adults were smeared on glass slides and preparations were stained with an anti-*B. burgdorferi* isothiocyanate conjugated immunserum prepared according to Peacock *et al.* (1971). Adult ticks were also histologically inves-

tigated for *B. burgdorferi* infection using Dieterle staining as described by LEBET & GERN (1994).

Transmission of B. burgdorferi *to mice by transovarially infected ticks:*

Larval (F1 and F2) and nymphal (F1) ticks were fed on mice and the infection status of the mice was evaluated by borrelia isolation from ear biopsies, xenodiagnosis, or direct detection of spirochetes in the blood. Ear specimens were obtained from anesthetized animals by resection with surgical scissors after cleaning the ear with 70% ethyl alcohol. Biopsies were taken at various intervals for 97 days after tick infestation. The specimens were transferred into tubes containing the medium described by SINSKY & PIESMAN (1989). The cultures were scored by dark field microscopy after 10 days at 34°C.

To perform xenodiagnosis, uninfected *I. ricinus* larvae from a breeding laboratory colony free of borrelia infection were allowed to feed on mice at various intervals after tick challenge. Xenodiagnostic ticks were examined for *B. burgdorferi* after molting to nymphs.

Blood was taken from the retroorbital plexus of the mice. Plasma was obtained by centrifugation of EDTA-K blood at 2500 rpm in a microfuge as described by STANEK *et al.* (1986). Plasma was examined using dark field microscopy (magnification 500x).

Statistical analysis

Fisher's exact test was used to compare proportion of infected, systemically infected and transovarial transmission in engorged field-collected females and F1 females.

RESULTS

Transovarial transmission rates by field-collected females systemically infected.

We investigated the transovarial transmission rates in field-collected females presenting an ovary infection. A total of 447 field collected female *I. ricinus* which fed on rabbits were dissected after oviposition and investigated for spirochetes in their midgut and ovary (Table 1). Borreliae were found in the midgut of 65 ticks (14.5 %) and in 21 of these midgut infected ticks (32.3 %) borreliae were also present in the ovary (Table 1). Only 3/21 (14.3 %) females with ovary infection transmitted the microorganism to their progeny.

Transovarial and subsequent transstadial transmissions of B. burgdorferi

We were interested in following the maintenance of transovarially transmitted *Borrelia* through generations. For this purpose, the progeny of 358 field-collected *I. ricinus* females was examined either as eggs (n= 17620) or as larvae (n= 3546). The fre-

	Field-collected females	F1 females
Nb infected/examined females	65/447 (14.5%)	17/116 (11%)
Nb systemically infected/infected females	21/65 (32.3%)	14/17 (82.4%)
Nb systemically infected females which transovarially transmitted	3/21 (14.3%)	10/13 (76.9%)

Table 1: *Borrelia burgdorferi* infection in field-collected and F1 *I. ricinus* female ticks after egg-laying.

	Progeny of field collected females					Total
	no 1	no 2	no 3	no 4	no 5	
F1 eggs	24/55 (44%)	39/60 (65%)	74/115 (64%)	nd	65/65 (100%)	202/295 (69%)
F1 larvae	no hatching	17/36 (47%)	111/135 (82%)	100/106 (94%)	99/104 (95%)	327/381 (86%)
F1 nymphs		12/18 (67%)	112/132 (85%)	56/60 (93%)	94/120 (78%)	274/330 (83%)
F1 adults		2/16 (13%)	5/79 (6.3%)	3/24 (12,5%)	nd	10/119 (8.4%)

Table 2: Follow-up of *Borrelia burgdorferi* infection rate in the F1 generation of transovarially infected eggs, larvae, nymphs and adult *Ixodes ricinus* ticks

nd: not done

	Progeny of field collected females					Total
	no 1	no 2	no 3	no 4	no 5	
F2 egg laying	nd	2/6 (33.3%)	0/70 (0%)	8/25 (32%)	nd	10/109 (9%)
F2 eggs	nd	84/130 (65%)		452/520 (87%)	nd	536/650 (82.5%)
F2 larvae	nd	no hatching		244/264 (92%)	nd	

Table 3: Follow-up of *Borrelia burgdorferi* infection rate in the F2 generation of transovarially infected egg layings, eggs and larval *I. ricinus* ticks.

nd: not done

quency of transovarial transmission was low since only 5/358 females (1.4 %) transmitted *Borreliae* to the next generation (Table 2). However, 44 to 100% of egg or F1 larva batches were infected (Table 2). Infection was maintained transstadially since 83% F1 nymphs were found infected. No significant difference was observed between the infection rates of F1 larvae and F1 nymphs (p=0.4). In the derived unfed F1 adults, borreliae were detected in 10/119 adults (8.4%; 4 females and 6 males) (Table 2).

Systemic infection in F1 female ticks after oviposition

In F1 females, examined for borrelia infection after oviposition, borreliae were found in the midgut of 17/116 ticks (11 %) and 14 of these 17 midgut infected females (82.4 %) also presented an infection in the ovary (Table 1). Among these 14 ovary infected F1 females, 13 laid eggs and 10/13 (76.9%) egg layings were infected.

Infection rate of F1 females (11%) after egg laying was not significantly different from infection rate of field-collected females (14.5%) (p=1) (Table 1). However, systemic infections with ovary infection were more frequent in F1 females (82.4%) than in field-collected females (32.3%) (p=0.0003). Moreover, if ovary infection was present transovarial transmission was more frequent in F1 females (76.9%) than in field-collected females (14.3%) (p=0.0007) (Table 1).

B. burgdorferi *infection in the F2 generation*

A total of 109 F2 egg layings were investigated and 10 egg layings (9%) derived from the F1 progeny of field-collected females (nos 2 and 4) were infected (Table 3). F1

females (n=70) derived from field-collected female no 3 did not transmit *Borreliae* to F2 generation (Table 3) (a total of 4550 eggs were examined). The infection rates of F2 eggs of these 10 F2 egg layings varied from 65% to 87% and borreliae were observed in 92% of F2 larvae derived from the progeny of female no 4 (Table 3).

Transmission of B. burgdorferi sl *to mice by F1 larvae and nymphs and by F2 larvae*

To investigate if transovarially infected larvae and nymphs can transmit borreliae to hosts, 4894 F1 larvae and 695 F1 nymphs were fed on 28 and 25 mice, respectively. None of the xenodiagnostic ticks fed on these mice (n=1389) and none culture tube containing ear biopsies revealed *Borrelia* infection. However, examination of the plasma of 11 additional mice infested by 275 F1 nymphs showed that 9 mice were infected at day 15 after tick infestation.

To examine if F2 larvae were able to transmit borreliae to mice, 360 F2 larvae were fed on 6 mice. Examination of their plasma at day 5, 13, 18 and 22 after tick infestation revealed that all mice were infected at day 13 and that 1/6 mice was infected at day 18.

Western blot analysis of infected mice sera showed a stronger reaction against *B. afzelii* (strain NE 517) than against *B. burgdorferi* ss (strain B31) and *B. garinii*(strain NE4) antigens (data not shown).

DISCUSSION

Transovarial transmission of *B. burgdorferi* has been described in *I. ricinus* in Europe with usually low larval infection rates (Wilske *et al.*, 1987, Doby *et al.*, 1989, 1990, Jaenson *et al.*, 1989, Miserez *et al.*, 1990, Matuschka *et al.*, 1992, Rijpkema *et al.*, 1994, Steinbrink, 1994, Zhioua *et al.*, 1994, Kurtenbach *et al.*, 1995, Halouzka *et al.*, 1995, Rijpkema & Bruinink, 1996). Similar observations have been reported for *I. scapularis* in the USA (Bosler *et al.*, 1983, Steere *et al.*, 1983, Piesman *et al.*, 1986, Magnarelli *et al.*, 1987). This suggests that transovarial transmission is unfrequent in ticks or that the efficiency of transovarial transmission is low.

In the present study, only 1.4% (5/358) field-collected *I. ricinus* females transmitted spirochetes to the next generation showing that transovarial transmission of *B. burgdorferi* in *I. ricinus* is unfrequent. Similar data have been obtained by Burgdorfer *et al.* (1983) and Nefedova *et al.* (2004) with 1.1% *I. ricinus* females and 6.3% infected *I. persulcatus* females, respectively, passing spirochetes to their filial ticks. However, higher frequencies of transmission have been described by Craine (1994) and by Stanek *et al.* (1986) who observed that 3/30 and 3/10 *I. ricinus* females, respectively, transmitted *Borreliae* transovarially. In contrast absence of transovarial transmission has been reported in *I. ricinus* females infected by *B. afzelii* (Matuschka *et al.*, 1998). Similarly, Burgdorfer (1989) and Patrican (1997) reported no transovarial transmission in *B. burgdorferi* infected *I. scapularis* females. In *I. pacificus*, one study described that infected ticks passed spirochetes to their filial ticks (Lane & Burgdorfer, 1987) whereas another study reported no transmission (Schoeler & Lane, 1993). Negative findings were also reported for *I. persulcatus* and *I. ovatus* (Nakao & Miyamoto, 1992). In another tick species, *I. hexagonus*, however a high frequency of transovarial transmission of borrelia has been described (Toutoungi & Gern, 1993). The reasons for the differences observed remain unknown but may be related to *Borrelia* strains as discussed below..

In the present study, if transovarial transmission was not frequent in *I. ricinus*, the efficiency of this mode of transmission was rather high: 44-100% of the F1 eggs or larvae were infected. Similar data have been obtained by Burgdorfer *et al.* (1983) who showed that *I. ricinus* females passed spi-

rochetes to 60% and 100% of their eggs. In another study, a similar infection rate (100%) was observed in the F1 larvaeand nymphs from *I. ricinus* females (STANEK *et al.*, 1986). In *I. scapularis* filial infection rates of 3.3-27% were reported in F1 larvae (MAGNARELLI *et al.,* 1987). BURGDORFER *et al.* (1988) and LANE & BURGDORFER (1987) observed egg infection rates of up to 100% in *I. scapularis* and in *I. pacificus*, respectively.

In our work, we followed maintenance of transovarially acquired spirochetes until the second generation, and showed that F1 and F2 generations of ticks were able to transmit spirochetes to mice as observed by direct observation of spirochetes in the plasma of mice. To our knowledge, only one previous study (STANEK *et al.* 1986) demonstrated the ability of transovarially infected ticks to transmit *Borrelia* to hosts. Interestingly, these authors also observed spirochetes in the blood of mice. The reasons why we were unable to isolate spirochetes from ear biopsies of mice or to detect borrelia infection by xenodiagnosis in these infected mice remain unknown but this could be due to the *Borrelia* species involved and possibly to the invasive character of the strains (LAGAL, 2004).

Interestingly, ovaries of F1 *I. ricinus* females were significantly more often infected (82.4%) than ovaries of field-collected females (32.3%) although there was no significant difference in the midgut infection rates between field-collected and F1 females (11% and 14.5%, respectively). Moreover, F1 females with infection in their ovaries transmitted more frequently spirochetes to their filial generation (76.9%) than field-collected ticks with an ovary infection (14.3%). Whether this is due to the tick lineage or to the pathogen involved, possibly related to invasive character, awaits further investigations. However, it should be noted that dissemination of spirochetes in tick organs varies among isolates as observed in 2 previous studies (CRIPPA *et al.*, 2002, FINGERLE *et al.*. 2002) suggesting that the pathogen might be involved in this phenomenon.

The significance of the findings obtained in this study awaits further investigations as well as the identification of the factors which promote inherited infection.

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