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Early detection of *Borrelia burgdorferi* sensu lato infection in Balb/c mice by co-feeding *Ixodes ricinus* ticks

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Abstract

In Europe, *Borrelia burgdorferi* is transmitted by *Ixodes ricinus* to animals and human. When infected and uninfected ticks co-feed on a host, spirochetes are transmitted from ticks to animal and also to uninfected ticks. Here, we used uninfected ticks to co-feed with infected ticks on mice to evaluate this method to detect early infection in mice. A total of 128 mice were challenged by infected nymphs placed in capsules glued on the back of the mice. Three days later uninfected larvae were added in the capsule to co-feed with infected nymphs and were examined for *Borrelia* infection after natural detachment. Infection in mice was also determined by xenodiagnosis and by spirochete isolation from ear skin biopsy and back skin biopsy taken at the tick attachment site one month after infection. A total of 111 mice were found to be infected by at least one of these four methods. *Borrelia* infection was observed in 95% of mice by the co-feeding method, in 92% of mice by xenodiagnosis, in 69% and in 68% of mice by cultivation of ear and back skin biopsies, respectively. Our results demonstrate that the co-feeding method is a very sensitive method which can be used to detect very early infection in mice infected by tick bites.

Key words: *Ixodes ricinus* – early infection – *Borrelia burgdorferi* – co-feeding transmission

Introduction

In Europe, *Borrelia burgdorferi* sensu lato (sl) is mainly transmitted by the tick *Ixodes ricinus*. Five *Borrelia* species have been isolated from this tick: *B. burgdorferi* sensu stricto (ss), *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae* (Gern et al., 1999). Among them three are recognized as pathogenic for humans: *B. burgdorferi* ss, *B. garinii* and *B. afzelii*.

In the laboratory, various methods are used to determine *B. burgdorferi* sl infection in animals. Direct detection of the pathogen in vertebrate hosts consists in *Borrelia* cultivation, *Borrelia* observation or DNA detection in host tissues (Morrison et al., 1999; Limbach et al., 1999) or using xenodiagnosis (Gern et al., 1997; Hu et al., 2001). In this case, spirochetes are detected or isolated from uninfected ticks after they have fed on infected animals. Indirect detection of infection is done by immunological tests. These tests are dependent on their specificity and/or sensitivity (Schaible et al., 1991; Kurtenbach et al., 1994). All these methods request generalization of the infection and therefore request time

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before infection can be detected. In fact, analysis of the infection in animals after infected tick bite showed that *Borrelia* did not immediately disseminate from the inoculation site and remained localized (Shih et al., 1992; Gern and Rais, 1996).

When infected *I. ricinus* ticks feed on a vertebrate host, within 24 hours after the onset of the blood meal, *B. burgdorferi* sl spirochetes may be transmitted into the animal skin through saliva (Kahl et al., 1998; Crippa et al., 2002). It was demonstrated that as infected *I. ricinus* ticks and uninfected ticks co-feed at the same site on hosts, spirochetes are transmitted not only from ticks to animal but from infected ticks to uninfected ticks as well (Gern and Rais, 1996; Richter et al., 2002). In our study, we used uninfected ticks to co-feed with infectious ticks on laboratory mice in order to evaluate this method to detect early infection in mice.

Materials and methods

Ticks and mice

Ticks used to infect mice were field collected. *B. burgdorferi* infection rate in collected ticks was evaluated using the immunofluorescence (IF) test and *Borrelia* isolation as described below. *I. ricinus* xenodiagnostic ticks (larvae) used in this experiment were from a laboratory colony free of spirochetes maintained at the Institut de Zoologie, University of Neuchâtel (Switzerland) (Graf, 1978). Eight weeks old Balb/C female mice (IFFA CREDO, 69592 L'Arbresle Cedex France) were used.

Infection of mice and spirochete detection in mice

To infect mice, infected nymphs were placed in a capsule glued on the back of the mice as described by Mbow et al. (1994). Four methods were used to detect *Borrelia* infection in mice: *1)* by co-feeding ticks which fed together with infected nymphs. Thirty uninfected larvae were added in the capsule three days after attachment of nymphs and allowed to feed together with infected nymphs. Then engorged co-feeding larvae were prepared for detection of *Borrelia* infection 1 week after detachment (see below); *2)* by a xenodiagnosis performed with 50 uninfected larvae placed on the head of each mouse. The derived nymphs were examined for *Borrelia* infection as described below; *3)* by isolation of spirochetes from ear skin biopsy; and *4)* by isolation of spirochetes from back skin biopsy taken at the tick inoculation site. The last three methods were performed one month after infected nymph detachment. Mice were anaesthetized as described by Mbow et al. (1994). After cleaning ear and back with 70% ethanol, the skin samples from ear and back were put individually into culture tubes containing 4 ml of supplemented BSK medium as described by Sinsky and Piesman (1989).

Each tick was cut into two pieces. One half was examined by immunofluorescence using a fluorescein isothiocyanate-conjugated polyclonal antibody which was prepared from a pool of Lyme borreliosis patient sera and which detects all *Borrelia* species (Gern et al., 1999). The other half was used for *Borrelia* isolation. The halved tick was placed in a culture tube containing BSK medium (Sinsky and Piesman, 1989), incubated at 34 °C and examined by dark-field microscopy every 10 days for 2 months.

Detection of Borrelia DNA in culture tubes and characterization of *B. burgdorferi* isolates

Polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) analysis were used to characterize *Borrelia* isolates and to detect *Borrelia* DNA in ungrown culture tubes (Postic et al., 1994). The variable intergenic spacer between tandemly repeated 23S (*rrl*) – 5S (*rrf*) ribosomal genes of *B. burgdorferi* sl was used as a template for amplification. The pellet from 1 ml initial culture containing spirochetes detected by dark-field microscopy and from 4 ml initial culture containing no visible spirochetes were prepared for PCR assay as described by Humair et al. (1999). The PCR products were analyzed by the RFLP technique using MseI restriction endonuclease to identify the genospecies of *B. burgdorferi* isolates (Postic et al., 1994). All *Borrelia* isolates obtained from ticks and mouse tissues were characterized using PCR/RFLP. Culture tubes containing biopsies from mice were all screened for the presence of *Borrelia* DNA by PCR/RFLP even if no spirochetes were visible.

Statistic analysis

The Fischer' s exact test was used to compare the different methods for the detection of *Borrelia* infection in mice. To account for multiple tests the Bonferroni correction was applied and p values < 0.0125 were considered significant.

Results

Borrelia infection rate in *I. ricinus* nymphs used to infect mice

The evaluation of *B. burgdorferi* infection rate of nymphs was performed in a subset of 135 collected nymphs: 32/135 ticks (23%) were found infected by IF and *Borrelia* isolation. Twenty four *Borrelia* isolates were obtained and characterized as *B. burgdorferi* ss (1/24), *B. garinii* (6/24), *B. afzelii* (16/24) and *B. valaisiana* (1/24). To infect the mice ($n = 128$), fourteen nymphs (infection rate 23%) were placed in capsule on the back of each mouse.

Detection of *Borrelia* infection in mice

Borrelia infection was found in 111/128 mice by at least one of the four methods. We detected infection

Table 1. Detection of *Borrelia* infection in mice (n = 111) using four methods.

Method	Co-feeding ticks			Xenodiagnostic ticks			Ear biopsy		Back skin biopsy	
	IF+ culture[1]	IF	Culture[1]	IF+ culture[1]	IF	Culture[1,2]	Culture[1]	DNA detection[3]	Culture[1]	DNA detection[3]
Number of infected mice	77	12	16	58	37	7	41	36	18	57
Total	105			102			77		75	

[1] Tubes with grown spirochetes
[2] *Borrelia* isolation from xenodiagnostic ticks was performed only in 76/111 infected mice.
[3] Tubes with ungrown spirochetes

Table 2. Characterisation of *Borrelia* isolates obtained from co-feeding ticks, xenodiagnostic ticks and mouse tissue biopsies.

Borrelia sp	Co-feeding ticks	Xenodiagnostic ticks	Ear skin biopsy		Back skin biopsy	
	Culture[1]	Culture[1]	Culture[1]	DNA detection[2]	Culture[1]	DNA detection[2]
B. garinii	8	5	7	2	5	11
B. afzelii	61	54	31	34	11	45
B. burgdorferi	1	0	3	0	1	0
B. valaisiana	1	0	0	0	0	0
B. garinii + B. afzelii	7	4	0	0	0	1
B. burgdorferi + B. afzelii	5	1	0	0	0	0
Untypeable	10	1	0	0	1	0
Total	93	65	77		75	

[1] Tubes with grown spirochetes
[2] Tubes with ungrown spirochetes

in 105/111 (95%) using the co-feeding method and 102/111 (92%) using xenodiagnosis (Table 1). No significant difference was observed between the two detection methods ($p = 0.60$). *Borrelia* infection was detected by both IF and isolation in ticks fed on 77 mice, by IF only in ticks fed on 12 mice and by isolation only in ticks fed on 16 mice (Table 1). Isolates were obtained from co-feeding ticks fed on 93/105 mice (88.6%, Table 2). *B. afzelii* was the most frequent isolated species followed by *B. garinii* (Table 2). In addition, *Borrelia* species transmitted to co-feeding ticks by 10 mice could not be characterized (PCR amplification was unsuccessful) although these isolates grew in culture (Table 2).

Borrelia infection was detected by IF in xenodiagnostic ticks fed on 95/102 mice (93%) (Table 1). Isolates were obtained from xenodiagnostic ticks fed on 65/76 mice (86%, Table 2). Most mice transmitted *B. afzelii* to xenodiagnostic ticks, followed by *B. garinii* (Table 2). In addition, one mouse transmitted *Borrelia* sp to xenodiagnostic ticks which could not be characterized (PCR amplification was unsuccessful) although *Borrelia* grew in culture.

Detection of mouse infection by biopsies showed that more isolates were obtained from ear skin biopsies (n = 41) than from back skin biopsies taken at the tick inoculation site (thereafter mentioned as back skin biopsy) (n = 18, $p = 0.00025$) (Table 1). Comparison of isolation of *Borrelia* from ticks with that from host tissues showed a higher isolation rate from co-feeding ticks than from ear ($p = 1.45 \times 10^{-7}$) or back skin biopsies ($p = 2.2 \times 10^{-16}$), and a higher isolation rate from xenodiagnostic ticks than from ear ($p = 1.94 \times 10^{-5}$) or back skin biopsies ($p = 7.78 \times 10^{-15}$).

Considering the low isolation rates obtained with mouse tissues (Table 1), we analyzed cultures of ungrown *Borrelia* by PCR for *Borrelia* DNA: 75 cultures of ear biopsies and 97 cultures of back skin biopsies were analyzed by PCR. *Borrelia* DNA was detected in 36/75 (48%) tubes inoculated with ear biopsies and in 57/97 (58%) culture tubes containing back skin biopsies (Table 1). Thus, using PCR, the success of detection of *Borrelia* infection in mice increased from 41/111 (37%) to 77/111 (69%) for ear biopsies and from 18/111 (16%) to 75/111 (68%) for back skin biopsies. Using PCR, there was

no more significant difference between *Borrelia* infection detected by ear biopsy and by back skin biopsies (75/111, 68%) ($p = 0.89$). However, a higher detection rate of infection in mice was obtained from co-feeding ticks than from ear biopsies ($p = 1.02 \times 10^{-6}$) or from back skin biopsies ($p = 2.45 \times 10^{-7}$), and from xenodiagnostic ticks than from ear biopsies ($p = 3.07 \times 10^{-5}$) or back skin biopsies ($p = 8.72 \times 10^{-6}$).

B. afzelii and *B. garinii* were both detected in mouse cultures of ear and back skin biopsies. There was no significant difference between the two *Borrelia* species in ear and back skin, respectively. *B. burgdorferi* ss was detected in three culture tubes with ear biopsies and one culture tube with back skin biopsy (Table 2). Only one mixture of *B. afzelii* and *B. garinii* DNA was detected in a culture tube containing back skin biopsies.

Globally, in this experience the *Borrelia* species infecting 107/111 mice were identified: 79 mice (74%) were infected by one species which was identified in ticks or/and in mouse tissue. The remaining 28 mice (26%) were infected by more than one species. In only 4/111 infected mice the *Borrelia* species could not be determined. There was a good correlation between the *Borrelia* species observed in co-feeding ticks and those found in mouse tissues or xenodiagnostic ticks. In fact, in 78/83 (93%) mice the *Borrelia* species in co-feeding ticks was the same as the ones identified in mouse tissues or xenodiagnostic ticks.

Discussion

The most important finding of this study is that *Borrelia* infection in mice infected through *I. ricinus* tick bites can be detected very early, i.e. 3–4 days after the infectious blood meal. In fact, a co-feeding transmission occurred between infected ticks and uninfected ticks allowing very early detection of infection in 95% of infected mice, in the week following the infectious tick blood meal. The identification of *Borrelia* species in co-feeding ticks correlated well (93%) with the *Borrelia* species identified in mice as detected by tissue biopsies or by xenodiagnosis.

Co-feeding transmission of *Borrelia* has been described for *B. burgdorferi* ss in *I. ricinus* (Gern and Rais, 1996), *B. afzelii* in *I. ricinus* (Richter et al., 2002), *B. burgdorferi* ss in *I. scapularis* (Patrican, 1997; Piesman and Happ, 2001) and *B. garinii* in *I. persulcatus* (Sato and Nakao, 1997). Here, *B. burgdorferi* ss, *B. afzelii* and *B. garinii* were identified in co-feeding *I. ricinus* ticks fed on mice. However, since co-feeding ticks were examined for *Borrelia* infection as fed ticks it is not known whether these three *Borrelia* species can survive the moult after a blood-meal on mice. Further studies are needed to answer this question. *B. valaisiana* was detected in co-feeding ticks in one mouse, but not in mouse tissues or in xenodiagnostic ticks. Apparently, *B. valaisiana* was unable to survive in the mouse which could be explained by the fact that this *Borrelia* species has been found in nature associated with birds and never with rodents (Humair et al., 1998; Kurtenbach et al., 1998).

Xenodiagnosis allowed to detect infection in 92% of infected mice one month after bites by infected ticks. This method is as sensitive as the detection of infection by co-feeding ticks but can be used only when infection has disseminated and has been established in the host that is about one month after the infectious tick blood meal (Gern and Rais, 1996).

Classical methods to detect infection in laboratory animals by isolation of *B. burgdorferi* sl from host tissue were also used: 37% of infection in mice were observed by isolation from ear biopsy samples and 16% from back skin biopsy samples taken at the tick inoculation site. This demonstrates that *B. burgdorferi* sl detection by *Borrelia* isolation from back skin biopsies taken at the tick inoculation site is less efficient than from ear biopsies. It is known that the success of *Borrelia* isolation may depend on the number of spirochetes inoculated in culture tubes (Gern et al. 1999; Hu et al., 2001). Here we could not observe whether the tick inoculation site (back skin biopsy) was infected by less spirochetes than the ear since no histological analysis or quantitative PCR was done on mouse tissues. However, we observed that it was easier to obtain *Borrelia* isolates from ear biopsies than from back skin biopsies. Using PCR assays, *Borrelia* DNA was detected in culture medium containing host tissues and this increased the success of *Borrelia* detection from 37% to 69% for ear biopsies and from 16% to 68% for back skin biopsies. The difference in success of isolation between ear biopsy and back skin biopsy may reflect the adaptation of spirochetes to vertebrate host tissue: spirochetes isolated from back skin are in the host for a few hours whereas spirochetes from ear biopsy had time to disseminate. This shows the importance of using PCR on culture medium containing host tissues when no grown spirochetes are observed. Isolation of *B. burgdorferi* sl from ear tissue appears, here, less sensitive (37%), than described for *B. burgdorferi* ss by Sinsky and Piesman (1989) who described an isolation rate from ear biopsy ranging from 92% to 100%. This is probably

due to the *Borrelia* species isolated from mouse tissue in the BSK medium. In fact the only species which did not benefit from the use of the PCR in our study was *B. burgdorferi* ss. In fact *B. burgdorferi* ss was always easily cultured from host tissues whereas *B. afzelii* was the species which was the most often detected by PCR and which appeared as the more difficult to isolate from host skin. Humair et al. (1999) also had difficulties to isolate *B. afzelii* from rodent skin (8.3% – 27.6%) whereas isolation from xenodiagnostic ticks was higher (44% – 51.4%). The reason for this remains unknown.

In conclusion, we demonstrated that *Borrelia* infections in mice can be detected very early by co-feeding ticks. This method appears more rapid and effective than the other methods used in our study to detect *B. afzelii*, *B. garinii* and *B. burgdorferi* ss infections. It could be used to obtain early results on the infection status of mice in laboratory studies, for example by investigating the success of early treatment with antibiotics or in vaccination trials under the natural infection route through tick bites.

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