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Two ways of experimental infection of *Ixodes ricinus* ticks (Acari: Ixodidae) with spirochetes of *Borrelia burgdorferi* sensu lato complex

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Abstract. A previously reported procedure for the introduction of *Borrelia* spirochetes into tick larvae by immersion in a suspension of spirochetes was tested on *Ixodes ricinus* (L.) ticks and three of the most medically important European *Borrelia* genomic species, *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. The procedure was compared with "classical" infection of nymphs by feeding on infected mice. Both methods yielded comparable results (infection rate 44–65%) with the exception of *B. afzelii*, which produced better results using the immersion method (44%) compared with feeding on infected mice (16%). Nymphs infected by the immersion method at the larval stage were able to transmit the infection to naïve mice as shown by serology and PCR detection of spirochetal DNA in organs. The immersion method is faster than feeding on infected mice and provides more reproducible conditions for infection. It can be exploited for studies on both pathogen transmission and *Borrelia*-vector interactions.

In Central Europe, *Ixodes ricinus* (L.) represents the principal vector of human pathogens such as *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and tick-borne encephalitis virus (Brouqui et al. 2004, Charrel et al. 2004). In Europe, prevalence rates of *B. burgdorferi* sensu lato in host-seeking nymphs and adults vary between 2% and 43% and 3% and 58% respectively (Hubálek and Halouzka 1998). Transovarial transmission of spirochetes is possible, but rare (Zhioua et al. 1994).

According to studies on the main American vector of *Borrelia* spirochetes, *Ixodes scapularis*, the larvae ingest spirochetes from infected rodents. This results in a population of spirochetes in the midgut, which is transmitted to the nymphal stage. Upon feeding, spirochetes migrate to the salivary glands via the haemolymph and are transmitted to the new host with the saliva (Piesman et al. 1990, Piesman 1995, de Silva and Fikrig 1995). Transmission of a pathogen by tick feeding cannot be compared with intradermal injection of a similar number of spirochetes. First, a feeding tick delivers spirochetes continuously during the feeding period (Crippa et al. 2002) second, tick saliva affects the host immune system facilitating transmission of the pathogen (Nuttall and Labuda 2004).

In many types of experiments including assessment of vaccines against tick-borne pathogens, saliva-activated transmission of pathogens and pathogen-tick interactions, efficient and reproducible infection of all tick developmental stages is required. The standard system for infecting *I. scapularis* and *I. pacificus* ticks by feeding on infected hosts was developed by Piesman (1993). More recently, *I. scapularis* larvae were shown to be infected by immersion in cultures of *Borrelia* spirochetes (Policastro and Schwan 2003). We examined both methods using the European tick *I. ricinus* and three medically important *Borrelia* genomic species, *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*.

MATERIALS AND METHODS

Mice. SPF female C3H/HeN mice 8 to 12 weeks old were purchased from Charles River, Sulzfeld, Germany.

Ticks. *Ixodes ricinus* ticks from a laboratory colony at the Institute of Parasitology, Biology Centre AS CR, České Budějovice, negative in *Borrelia burgdorferi* sensu lato PCR screening, were used throughout the experiments.

Bacteria. The CB-43 strain of *B. afzelii* (Štěpánová-Tresová et al. 1999), the CB-53 strain of *B. burgdorferi* sensu stricto and the CB-61 strain of *B. garinii* were isolated from *I. ricinus* ticks in the South Bohemia region and identified using genomic species-specific PCR. The spirochetes were grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma) supplemented with 6% rabbit serum at 34°C. Low-passage spirochetes were used (2 to 7 passages).

Infection of ticks by feeding on infected mice. Groups of five mice were each inoculated subcutaneously in the dorsal thoracic midline with 10^3 spirochetes of either *B. burgdorferi* sensu stricto (strain CB-53), *B. afzelii* (CB-43) or *B. garinii* (CB-61) respectively in 0.1 ml of BSK-H medium per mouse. One month post infection, groups of 20 nymphs were placed into retaining cells attached to the back of each mouse. Control nymphs were fed on mock-infected mice. After full engorgement the nymphs were maintained at 25°C and a relative humidity 80% until metamorphosis. Ten adults from each mouse were examined for the presence of spirochetes using PCR.

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Infection of ticks by immersion in cultures of spirochetes. Groups of circa 40 larvae were placed into microcentrifuge tubes (n = 3, one tube for each genomic species) with 0.5 ml of spirochete suspension containing 10^7 cells per ml and incubated at 32°C for 2 h. Tubes were gently vortexed every 10 min. The control group of 40 larvae was immersed in plain BSK-H medium. Then, the larvae were washed briefly in 70% ethanol and twice in distilled water and placed on mice to complete engorgement. After moulting, 20 nymphs were examined for the presence of each spirochete genomic species by PCR.

Detection of *B. burgdorferi* sensu lato DNA by PCR. Detection of spirochetal DNA was performed in ticks after moulting for two reasons. First, engorged blood can interfere with PCR; second, the trans-stadial transmission of spirochetes could be determined. JETquick Tissue DNA Spin Kit (Genomed) was used for DNA isolation. Each tick was homogenized in 30 µl of PBS and the supernatant was transferred into a microcentrifuge tube with 200 µl of T1 buffer and 25 µl of proteinase K (20 mg/ml, Genomed). Samples were incubated at 56°C overnight. Additional steps were performed according to the manufacturer's instructions. For DNA isolation from mouse organs, samples with mass of 15–20 mg were homogenized in 200 µl of T1 buffer with 25 µl of proteinase K; further processing was as for tick homogenates. A segment of the *B. burgdorferi* sensu lato 16S rRNA gene (669 bp) was amplified using forward primer 5'-ACG CTG GCA GTG CGT CTT AA-3'and reverse primer 5'-CTG ATA TCA ACA GAT TCC ACC C-3' (Liebisch et al. 1998). Primers were produced by Generi-Biotech, Czech Republic. PCR was performed in a reaction volume 20 µl containing 2.0 µl of processed DNA sample, 2.0 µl of Taq buffer (Generi-Biotech), 1.6 µl of 10 mM dNTP each (TopBio), 1.0 + 1.0 µl of 0.01 mM oligonucleotide primers, 0.1 µl (2 U) Taq DNA polymerase (Generi-Biotech), 12.3 µl of H_2O. PCR was performed in an UNO II thermocycler (Biometra). Each sample (after initial DNA denaturation at 95°C for 5 min) was amplified for 35 repeated cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, chain elongation at 72°C for 1 min. PCR amplification products were resolved on a 1% agarose electrophoresis gel and visualised under UV light with SYBR Green II (Sigma).

Ability of infected ticks to transmit the infection to mice. Groups of 20 larvae were placed on naïve C3H/HeN mice immediately after immersion in the suspension of spirochetes and left to feed to repletion. Two mice were used for each *Borrelia* genomic species. Nymphs infected as larvae by immersing in the suspension of *B. afzelii* were placed in groups of five on four naïve C3H/HeN mice and left to feed to repletion. Two control mice were exposed to nymphs developed from mock-infected larvae. One month later the mice were examined by serology and PCR detection of spirochetes in the heart and urinary bladder was also performed.

ELISA procedure. Wells of high-binding, F96 Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with the sonic extract from *B. burgdorferi* sensu stricto strain CB-53 (100 µl per well) overnight at 4°C. The antigen was diluted in a carbonate buffer (pH 9.6) to a concentration 10 µg/ml. After blocking with PBS and 10% newborn calf serum (NCS) for 2 h at room temperature, mouse sera diluted 1:200 in PBS were added (50 µl per well) and incubated overnight at 4°C. Plates were then incubated with peroxidase-conjugated swine anti-mouse IgG (Sevak, Prague, Czech Republic) diluted 1:2000 in PBS for one hour at 37°C. An enzymatic colour reaction was generated using orthophenylendiamine substrate, stopped after 10 min with 2M H_2SO_4 and measured at 490 nm with an ELISA spectrophotometer (Multiskan MCC/340, Labsystems Oy, Helsinki, Finland). After each incubation the plate was washed at least three times with 0.05% Tween 20 in PBS. The cut off value was calculated as double the OD value for control sera from non-infected mice.

RESULTS

Infection of ticks by feeding on infected mice

Of 15 mice in groups of five, each infected with one of three genomic species of *B. burgdorferi* sensu lato, almost all mice were seropositive in ELISA one month post infection. Although all sera were tested with the antigen from *B. burgdorferi* sensu stricto (strain CB-53), the OD exceeded the cut off value due to antigenic cross-reactivity between all three genomic species used (data not shown). *Ixodes ricinus* nymphs were placed on each mouse and after full engorgement and moulting, adults were examined for the presence of spirochetes by PCR. Table 1 shows the results with ticks infected with particular genomic species of *B. burgdorferi* sensu lato complex. Ticks feeding on all five mice infected with *B. burgdorferi* sensu stricto became infected with the infectivity rate ranging from 20% to 100%. Taken together, spirochetal DNA was demonstrated in 32 of 50 ticks, which represents 64%. Infection of ticks feeding on mice infected with *B. afzelii* was less efficient. None of the ticks feeding on mice without *Borrelia*-specific antibodies became infected. The infectivity rate of ticks that had fed on seropositive mice ranged from 0% to30% with the average 16% (including ticks feeding on

Table 1. Infection of *Ixodes ricinus* nymphs by feeding on mice infected with *Borrelia burgdorferi* sensu stricto (strain CB-53), *Borrelia afzelii* (CB-43) and *Borrelia garinii* (CB-61).

	Mouse No.	Seroconversion	Infection rate	
			Infected/Total	%
B. burgdorferi s.s.	1	+	10/10	100
	2	+	7/10	70
	3	+	9/10	90
	4	+	2/10	20
	5	+	4/10	40
		Total	32/50	64
B. afzelii	1	+	2/10	20
	2	+	3/10	30
	3	–	0/10	0
	4	+	3/10	30
	5	+	0/10	0
		Total	8/50	16
B. garinii	1	–	0/10	0
	2	+	8/10	80
	3	+	4/10	40
	4	+	5/10	50
	5	+	5/10	50
		Total	22/50	44

Table 2. Infection rate of *Ixodes ricinus* nymphs infected at the larval stage by immersion in suspension of three genomic species of *Borrelia burgdorferi* sensu lato.

Borrelia genomic species	Infection rate	
	Infected/Total	%
B. burgdorferi s.s.	13/20	65
B. afzelii	8/20	40
B. garinii	10/20	50
Total	321/60	52

Table 3. Infectivity to mice of nymphs infected at the larval stage by immersion in suspension of *Borrelia afzelii.*

Mouse No.	Seroconversion	Spirochetes in organs*	
		Hearth	Urinary bladder
1	+	+	+
2	–	–	+
3	–	–	–
4	+	+	+
5**	–	–	–
6**	–	–	–

*Spirochetal DNA was detected by PCR; **Control mice infested with *Borrelia*-free nymphs.

the seronegative mouse). Together 44% of nymphs were infected by feeding on mice infected with *B. garinii*. One mouse was seronegative with 0% of infected nymphs; the infection rate for the remaining four mice ranged from 40% to 80%.

Regardless of the *Borrelia* genomic species, a total of 61/150 nymphs (41%) became infected. The highest tick infection rate was achieved with *B. burgdorferi* sensu stricto, the lowest with *B. afzelii*.

Infection of ticks by immersion in a suspension of *Borrelia* spirochetes

Ixodes ricinus larvae were immersed in the suspension of one of three *Borrelia* genomic species. After moulting to nymphs, they were examined for the presence of spirochetes by PCR. The results are summarized in Table 2. The most efficient infection (65% of infected ticks) was achieved with *B. burgdorferi* sensu stricto (strain CB-53). *Borrelia garinii* was more infective than *B. afzelii* (50% versus 40% of infected ticks).

Ability of infected ticks to transmit infection to the new host

Mice on which the larvae were placed immediately after immersion in the suspension of spirochetes, were examined for the presence of spirochetal DNA or *Borrelia*-specific antibodies one month post engorgement. No evidence of transmission of *Borrelia* spirochetes from the larvae to mice was observed.

To demonstrate the ability of immersion-infected larvae to maintain *B. afzelii* infection trans-stadially and to transmit the spirochetes to the host, moulted nymphs were fed on naïve mice. One month post engorgement specific antibodies were detected in two of four mice. Further examination of tick-exposed mice by PCR showed spirochetal DNA in the heart and urinary bladder of both seropositive mice and in the urinary bladder of one of seronegative mice. The fourth mouse and both control mice were negative in the PCR assay (Table 3).

DISCUSSION

The purpose of this study was to examine two methods of experimental infection of *Ixodes ricinus* ticks with three genomic species from the *Borrelia burgdorferi* sensu lato complex pathogenic for humans. We exploited a standard method of tick infection by feeding on infected mice (Piesman 1993) and the more recently developed method based on immersion of tick larvae in a suspension of spirochetes (Policastro and Schwan 2003). The first procedure resembles the natural route of infection. Mice can be infected intradermally or subcutaneously or via feeding of infected ticks (Piesman 1993) or by inoculation of spirochetes mixed with tick salivary gland extract (Pechová et al. 2002). Another factor which can influence the success of host-to-tick transmission is the infectious dose used for host infection. While Piesman (1993) obtained a tick infection rate ranging between 18% and 83% for three strains of *B. burgdorferi* sensu stricto, using inoculum of 10^7 spirochetes per host, we achieved a rather high infection rate (64%), using mice infected with a much lower dose (10^3 spirochetes per mouse).

The different infection rates obtained for particular *Borrelia* genomic species cannot be connected with the vector competence of *I. ricinus* tick. In Europe, the most prevalent genomic species is *B. afzelii*, followed by *B. garinii*; *B. burgdorferi* sensu stricto is the least frequent (Rauter and Hartung 2005). The different infection rates obtained in our experiments may relate to the different virulence of the strains used, which can depend on the number of passages *in vitro*. Various isolates of the same *Borrelia* genomic species can vary markedly in their infectivity for ticks or hosts (Peavey and Lane 1996) and even one *Borrelia* strain can represent a heterogeneous mixture of clones varying in morphology, growth rate, protein profile, plasmid content and infectivity (Elias et al. 2002).

Infection rates achieved by immersion of *I. ricinus* larvae in the suspensions of *Borrelia* spirochetes were similar to infection rates of nymphs fed on infected mice. While *B. burgdorferi* sensu stricto was again the most infectious, the lowest infection rate was observed for *B. afzelii*. Values varied from 40% to 65% depending on the genomic species, compared with 16% to 64% for nymphs fed on infected mice. The percentage of nymphs infected with *B. burgdorferi* sensu stricto (65%) was identical with the infection rate of *I. scapularis* larvae immersed in a suspension of spirochetes of the same genomic species (Policastro and Schwan 2003). Policastro and Schwan (2003) also demonstrated that immersion-infected larvae were able to transmit the pathogen to the host immediately after immersion. This transmission was not demonstrated in our experiments.

Mice on which immersion-infected larvae were placed to complete engorgement, did not seroconvert and *Borrelia* DNA was not detected in their organs by PCR. The difference between our experiments and those by Policastro and Schwan (2003) consists in "holding time", the time for which the larvae were allowed to recover after immersion in the suspension of spirochetes before being applied to mice. While the holding time used by Policastro and Schwan was 18 h to 3 days, in our design the larvae were placed on mice immediately after immersion. This way the engorgement efficiency could be increased to 50–70% (data not shown) compared with 10–50% reported by Policastro and Schwan (2003). On the other hand, spirochetes in larvae placed on mice without a holding time could have not enough time to adapt to the inner tick milieu to be able to pass from the midgut to salivary glands and infect the host.

To show that nymphs moulted from immersion-infected larvae are able to infect the host, nymphs infected with *B. afzelii* (infection rate 40%) were placed on C3H/HeN mice in groups of five and allowed to feed to full engorgement. Three of four mice became infected as demonstrated by detection of spirochetal DNA in the heart and urinary bladder. One of these infected mice was negative in ELISA, which confirms the limitations in serology testing as a method for demonstrating *Borrelia* infection (Tylewska-Wierzbanowska and Chmielewski 2002).

The present work shows that the procedure of tick infection by immersion in a suspension of *Borrelia* spirochetes can be successfully exploited for the infection of *I. ricinus* with the three most important European genomic species of the *B. burgdorferi* sensu lato complex. This method is faster than feeding on infected mice and yields comparable infection rates. Although representing an artificial method of tick infection, like capillary feeding (Fingerle et al. 2002), it provides more reproducible conditions for infection and the following life stages (nymph and adult) can be used for studies on pathogen transmission to naïve and tick- or pathogen-immune hosts as well as for the analysis of *Borrelia*-vector interactions.

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