Visualization of *Borrelia burgdorferi sensu lato* by fluorescence *in situ* hybridization (FISH) on whole-body sections of *Ixodes ricinus* ticks and gerbil skin biopsies

Bettina Hammer,¹ Annette Moter,¹ Olaf Kahl,² Gerd Alberti³ and Ulf B. Göbel¹

Author for correspondence: Bettina Hammer. Tel: +49 30 450 524074. Fax: +49 30 450 524902.
e-mail: bettina.hammer@charite.de

¹ Institut für Mikrobiologie und Hygiene, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Dorotheenstraße 96, D-10117 Berlin, Germany

² Institut für Biologie, Angewandte Zoologie/Ökologie der Tiere, Freie Universität Berlin, Haderslebener Straße 9, D-12163 Berlin, Germany

³ Zoologisches Institut und Museum, Ernst-Moritz-Arndt-Universität, J.-S.-Bachstraße 11/12, D-17487 Greifswald, Germany

The objective of this study was to visualize borreliae directly in whole-body sections of *Ixodes ricinus* by fluorescence *in situ* hybridization (FISH). *Borrelia afzelii* mono-infected or *Borrelia burgdorferi sensu stricto* (ss)/*B. afzelii* double-infected nymphs were fixed, embedded in cold polymerizing resin and sectioned. The same sample processing was applied to skin biopsies taken from a Mongolian gerbil after an infectious tick-bite. FISH was carried out using 16S-rRNA-directed, fluorescence-labelled oligonucleotide probes specific for the genus *Borrelia* and specific within the group of Lyme borreliosis-associated genospecies *B. afzelii*, *B. burgdorferi* ss, *Borrelia garinii* and *Borrelia valaisiana*. Sensitivity and specificity of the newly designed probes were evaluated using PCR, dot-blot hybridizations and FISH. Despite significant autofluorescence of certain tick tissues (which allowed good histological orientation within the sections), borreliae showing the typical spirochaetal morphotype were clearly visible in five out of six putatively infected ticks. These findings were confirmed by electron microscopy of ticks from the same infected batch as used for FISH. Attempts to produce ticks infected by two different *Borrelia* genospecies were not successful. FISH on whole-body sections of resin-embedded ticks offers the possibility of visualizing and identifying borreliae within tick tissues. This technique has great potential for the investigation of the transmission of bacteria or other micro-organisms by arthropod vectors. Furthermore, clear visualization of single spirochaetes distributed along subcutaneous fat cell membranes in gerbil skin biopsies suggests that FISH might also be suitable for the detection of borreliae in clinical tissue specimens.

Keywords: Lyme borreliosis, arthropods, mixed infection, *in situ* hybridization, FISH

INTRODUCTION

Borrelia burgdorferi sensu lato (sl), causing Lyme borreliosis (LB) is transmitted by and to *Ixodes* ticks (Acari, Ixodidae) during their blood meals on vertebrate hosts. Three *Borrelia* genospecies, *Borrelia afzelii*, *Borrelia garinii* and *B. burgdorferi sensu stricto* (ss) cause human infection and disease. The pathogenicity of *Borrelia valaisiana* for humans is assumed (Rijpkema *et*

al., 1997). Each of these bacteria is transmitted by *Ixodes ricinus*, the main vector tick in Europe. In contrast, in North America, human LB seems to be caused exclusively by *B. burgdorferi* ss transmitted by *Ixodes scapularis* in the East and *Ixodes pacificus* in the West. Although the general borrelial migration pattern within infected *Ixodes* ticks during feeding from the tick midgut via the salivary glands into the vertebrate host seems to be the same in *I. ricinus* and *I. scapularis* (Benach *et al.*, 1987; Burgdorfer *et al.*, 1989; Piesman *et al.*, 1991; Piesman, 1995; Gern *et al.*, 1996), recent investigations addressing the risk of *B. burgdorferi* sl transmission by these tick species revealed intriguing differences. It was

Abbreviations: EM, electron microscopy; FISH, fluorescence *in situ* hybridization; IFA, indirect immunofluorescence assay; LB, Lyme borreliosis; sl, *sensu lato*; ss, *sensu stricto*.

shown that nymphal *I. ricinus* can transmit borreliae earlier during the blood meal than has been known from *I. scapularis* nymphs (Kahl *et al.*, 1998a). Interestingly, borreliae were found distinctly more often in the salivary glands of unfed *I. ricinus* than in unfed *I. scapularis* (Leuba-Garcia *et al.*, 1994; Lebet & Gern, 1994; Korenberg & Moskvitina, 1996). The reason(s) for these differences are as yet unknown and need further investigation.

The aim of the present study was to visualize borreliae in sections of infected vector ticks to show their spatial distribution. This was achieved by fluorescence *in situ* hybridization (FISH) using Cy3-labelled oligonucleotide probes. FISH allows simultaneous visualization and identification of micro-organisms in their natural micro-habitat. This method has been applied to a variety of environmental and clinical specimens (Amann *et al.*, 1996; Snajdr *et al.*, 1997; Schuppler *et al.*, 1998; Trebesius *et al.*, 1998; Fritsche *et al.*, 1999; for a review see Moter & Göbel, 2000). There have also been reports on its use to identify bacteria within infected tissues (Forsgren *et al.*, 1994; Berlau *et al.*, 1998; Trebesius *et al.*, 1998; Jansen *et al.*, 1999; Harmsen *et al.*, 2000; Hogardt *et al.*, 2000). Recently, FISH was applied to biopsies of digital dermatitis lesions embedded in cold polymerizing resin, allowing sectioning of the tissue with excellent histological conservation (Moter *et al.*, 1998). We adapted this protocol for use on ticks and designed oligonucleotide probes specific within the genus *Borrelia* and within the group of LB-associated genospecies *B. afzelii*, *B. burgdorferi* ss, *B. garinii* and *B. valaisiana*. PCR, dot-blot hybridization and FISH were performed to test the respective probes. This is the first report to investigate the spatial distribution of borreliae in whole-body sections of *I. ricinus* by FISH. To evaluate the newly developed technique, individuals from the same tick batch were investigated by indirect immunofluorescence assay (IFA) and electron microscopy (EM). To prove suitability of the procedure for clinical samples, we also applied the method to a skin biopsy of a Mongolian gerbil, previously infected by *B. afzelii* via tick-bite.

METHODS

Bacterial strains. *B. afzelii* strains AZ/2 and AZ/10 were originally from field-collected *I. ricinus* nymphs and were subsequently maintained by alternating between Mongolian gerbils (*Meriones unguiculatus*) and ticks for 5 years (four gerbil passages) and 3.5 years (two gerbil passages), respectively. *B. burgdorferi* ss-infected *I. scapularis* nymphs were field-collected in Westchester county (New York State, USA) and kindly provided by D. Fish (Yale School of Medicine, Epidemiology and Public Health, New Haven, CT, USA). The identity of the respective borrelial strains was confirmed by PCR and dot-blot hybridizations. Strain AZ/2 was identified by PCR as *B. afzelii* by S. Rijpkema (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) (Rijpkema *et al.*, 1995). In addition, *Borrelia* isolates were obtained from individual ticks cultured in BSK-H medium (Sigma-Aldrich). OspA serotyping using these isolates was carried out by B. Wilske (Max von Pettenkofer

Institute for Hygiene and Medical Microbiology, Munich, Germany) to confirm the strains as *B. afzelii* or *B. burgdorferi* ss (Wilske *et al.*, 1993).

The following cultured *Borrelia* and *Treponema* strains were included as controls in FISH [cells fixed in PBS (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄) with 3.7% (v/v), formaldehyde] and PCR experiments: *B. burgdorferi* ss (B31^T; tick isolate, USA, ATCC 35210), *B. garinii* (A; tick isolate, R. Ackermann, University Hospital of Cologne, Cologne, Germany), *B. afzelii* (PKo; human skin isolate, B. Wilske), *B. valaisiana* (VS116^T; tick isolate, Switzerland, kindly provided by D. Postic, Institut Pasteur, Paris, France), *Borrelia hermsii* (HS1^T; tick isolate, USA) and *Treponema denticola* (ATCC 33521).

Ticks and tick infection. Two batches of laboratory-reared proven *Borrelia*-negative *I. ricinus* ticks (second laboratory generation) were each *in vivo*-infected during their larval blood meal on a Mongolian gerbil previously fed upon by four *I. ricinus* nymphs infected with the local *B. afzelii* strains AZ/10 and AZ/2. Briefly, four infected ticks were placed onto each gerbil to feed to repletion. Two to three weeks after gerbil infection, approximately 100 unfed proven *Borrelia*-negative *I. ricinus* larvae (at least second laboratory generation) were fed on each gerbil. The fully engorged larvae were allowed to molt to the nymphal stage under quasi-natural temperature and photoperiodical conditions and were analysed for borreliae by IFA some weeks or months after nymphal ecdysis. To produce *I. ricinus* nymphs infected both with *B. afzelii* and *B. burgdorferi* ss, tick-naïve gerbils were infected by feeding of *I. ricinus* nymphs infected with *B. afzelii* strain AZ/2 and 27 d later feeding of *B. burgdorferi* ss-infected *I. scapularis* nymphs. In a second experiment, the sequence of infection was reversed: feeding of *B. burgdorferi* ss-infected *I. scapularis* nymphs and 45 d later feeding of *B. afzelii* (strain AZ/2)-infected *I. ricinus* nymphs. Subsequently, larval xenodiagnosis was performed on each gerbil to produce double-infected *I. ricinus* ticks. To evaluate the tick infection rate, 10 individuals of each resultant nymphal batch were screened for borreliae by IFA as described by Kahl *et al.* (1992) using a polyclonal antibody (rabbit hyperimmune serum against the *B. garinii* strain 1B29, kindly provided by A. Schönberg, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Berlin, Germany). Six resultant xenodiagnostic *I. ricinus* nymphs were analysed by PCR and dot-blot for the identification of mixed infections with *B. afzelii* and *B. burgdorferi* ss.

Gerbil skin biopsy. A skin punch biopsy from a gerbil was taken at the attachment site of a single *I. ricinus* nymph within the first 15 h after drop-off. This nymph belonged to the same *B. afzelii*-infected tick batch as used for FISH experiments (strain AZ/10). A second skin biopsy was taken in a distance of 1.5 cm from the tick-bite site as a putative negative control.

PCR. To identify the *Borrelia* genospecies and to detect *Borrelia* double infections within the produced tick batches, ticks were analysed by PCR using *Borrelia* genus-specific amplification of 16S rDNA and subsequent dot-blot hybridization. Ticks were individually processed for PCR by washing them twice in sterile distilled water and crushed in 100 µl 12.5% (w/v) Chelex 100 (Bio-Rad), as described by Maiwald (1996) with some modification. In brief, tubes with the crushed ticks were heated at 56 °C for 15 min and at 100 °C for another 10 min, vortexed thoroughly and chilled on ice. The samples were then centrifuged at 7000 *g* for 12 min and 5 µl of the supernatant was directly used for PCR or stored at -80 °C until further use. DNA preparation from cultured borreliae or treponemes was carried out according to standard protocols (Sambrook *et*

Table 1. Oligonucleotide probes

Oligonucleotide probe	Standardized probe name*	Specificity	Sequence (5'-3')	Position†	Label
EUB338	S-D-Bact-0338-a-A-18	<i>Bacteria</i>	GCTGCCTCCCGTAGGAGT	338–355	Fluorescein
Borr4	S-G-Borr-1068-a-A-21	Genus <i>Borrelia</i>	CCAACACCTCACAGCACGAGC	1068–1088	Fluorescein, Cy3
BaV1	S-LB-Ba-80-a-S-22	<i>B. afzelii</i>	GCAATACATTTAGTGGCGAACG	80–101	Digoxigenin
reBaV1	S-LB-Ba-80-a-A-22	<i>B. afzelii</i>	CGTTCGCCACTAAATGTATTGC	80–101	Cy3
BbV1	S-LB-Bb-80-a-S-19	<i>B. burgdorferi</i> ss	GCAATACATTCAGTGGCGA	80–98	Digoxigenin
reBbV1	S-LB-Bb-80-a-A-21	<i>B. burgdorferi</i> ss	GTTCGCCACTGAATGTATTGC	80–100	Fluorescein, Cy3
BgV1	S-LB-Bg-117-b-S-21	<i>B. garinii</i>	GATGATCTACCTACGAGATGG	117–137	Digoxigenin
reBgV1	S-LB-Bg-117-b-A-21	<i>B. garinii</i>	CCATCTCGTAGGTAGATCATC	117–137	Cy3
BV	S-LB-Bv-77-a-S-21	<i>B. valaisiana</i>	GTAGTAATACATCTAGTGGCG	77–97	Digoxigenin
reBV	S-LB-Bv-77-a-A-21	<i>B. valaisiana</i>	CGCCACTAGATGTATTACTAC	77–97	Cy3

* Standardized probe names according to Alm *et al.* (1996). LB indicates group of Lyme-borreliosis-associated borreliae.

† Numbering according to *E. coli* 16S rRNA.

al., 1989). After comparative sequence alignment (see below) a primer set for amplification of *B. burgdorferi* sl 16S rDNA was designed: Borr0 (5'-GGC-AGT-GCG-TCT-TAA-GCA-TGC-3', corresponding to position 42–62 in the *Escherichia coli* 16S rRNA gene) and Borr4 (5'-CCA-ACA-CCT-CAC-AGC-ACG-AGC-3', corresponding to position 1068–1088). To check its suitability for PCR experiments we used the program OLIGO (version 4.0). The PCR reaction was performed in a 50 µl mixture containing 30 mM Tricine, 50 mM KCl, 2.5 µg acetamide according to Barns *et al.* (1994), 15 pmol of each primer (Tip Molbiol), 0.05 mM of each nucleotide (Pharmacia), 2 mM MgCl₂ and 1.25 U AmpliTaq polymerase (Perkin-Elmer). Concentrations of MgCl₂, primers, nucleotides and the amplification protocol were optimized as described by Cobb & Clarkson (1994). Amplification conditions consisted of an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of 92 °C for 1.5 min, 62 °C for 1.5 min and 72 °C for 2 min, and a final extension step at 72 °C for 5 min. The amplification products were detected by agarose gel electrophoresis (1.5%). To assess the sensitivity of the PCR reaction, serial dilutions of template DNA ranging from 10 ng to 1 fg were produced. In addition, the cell content of borrelial cultures (strains A, PKo, B31^T and VS116^T) was examined by dark-field microscopy using a counting chamber (Neubauer). Cultures were diluted in double-distilled water to reach a number of 1000 spirochaetes per 10 µl and stored at –80 °C. Aliquots of this solution containing 1000 spirochaetes were added to separate DNA preparation tubes each containing a *Borrelia*-negative *I. ricinus* nymph and 12.5% (w/v) Chelex solution to a final volume of 100 µl. For PCR, 0.5, 2.5 and 5 µl of these Chelex-preparation supernatants was used, corresponding to 5, 25 and 50 spirochaetes, respectively. Both the cell counting procedure and PCR were repeated at least twice for each human-pathogenic *Borrelia* genospecies. The sensitivity of the method for mixed DNA templates of *B. burgdorferi* genospecies was tested for serial dilutions ranging from 100 to 1 pg DNA.

Oligonucleotide probes for FISH and dot-blot hybridization.

Five *Borrelia*-specific oligonucleotide probes directed to the 16S rRNA were designed after comparative sequence analysis of 16S rRNA sequences of respective type strains (*B. burgdorferi* sl) and/or well characterized strains of each genospecies, using the program CLUSTAL of the HUSAR 4.0 program package (Deutsches Krebsforschungszentrum, Heidelberg, Germany), (Table 1). Subsequently, selected

probes and primer sequences were compared to all 16S rRNA entries in the EMBL and GenBank databases (September 2000) using the FASTA program (HUSAR 4.0). The oligonucleotide sequences were further checked for their suitability by the program OLIGO 4.0. However, since each of these oligonucleotide probes except BV/reBV shows sequence homology to at least one non-target *Borrelia*-species within the *B. burgdorferi* species complex (Table 2), we describe the designed oligonucleotide probes as specific within the group of LB-associated genospecies. One exception was found: database analysis revealed homology between BgV1/reBgV1 and a single strain identified as *B. afzelii* (J1; GenBank accession no. L46697). However, at the probe position this strain is homologous to *B. garinii* published strains (e.g. accession nos X85198, X85193, X85199, D67018). In FISH experiments, the reverse primer Borr4 was used as an alternative genus-specific probe. In other experiments, it was added together with a LB-group-specific *Borrelia* probe to enhance signal intensities by multiple labelling according to Lee *et al.* (1993). All probes used for FISH were synthesized commercially and 5' end-labelled with fluorescein or Cy3 (indocarbocyanine; Interactiva). The fluorescein-labelled *Bacteria*-specific probe EUB338 (Amann *et al.*, 1990) (Tip Molbiol) was used as a control. To enhance the signal-to-noise ratio, we included a second green fluorescent dye, FluoX, for the labelling (5') of EUB338 (Interactiva). Probes for dot-blot hybridizations were labelled by adding digoxigenin-labelled nucleotides and terminal transferase according to the manufacturer's recommendations (Boehringer Mannheim; see Table 1).

Dot-blot hybridization. *Borrelia* genus-specific amplicons were analysed by dot-blot hybridization using digoxigenin-labelled oligonucleotide probes as described by Moter *et al.* (1998). Aliquots (5 µl) of the amplified products, including negative (PCR-negative control) and positive (*B. afzelii*, *B. burgdorferi* ss, *B. garinii* and *B. valaisiana*) controls, were transferred to positively charged nylon membranes (Boehringer Mannheim) and fixed by UV cross-linking. For subsequent use, filters were stripped by removing bound probes with 0.2 M NaOH/0.1% SDS, dried and stored at 4 °C.

FISH. Processing of biopsy specimens and fixed bacteria was carried out as described by Moter *et al.* (1998). A pre-treatment of the ticks was necessary. Putatively *B. afzelii*-positive ($n = 6$) and *Borrelia*-negative ($n = 5$) unfed *I. ricinus*

Table 2. Homology of the oligonucleotide sequences within the *B. burgdorferi* species complex

<i>Borrelia</i> genospecies	reBaV1	reBbV1	reBgV1	reBV	Borr4
<i>B. afzelii</i>	+	–	–	–	+
<i>B. burgdorferi</i> ss	–	+	–	–	+
<i>B. garinii</i>	–	–	+	–	+
<i>B. valaisiana</i>	–	–	–	+	+
<i>B. lusitaniae</i>	–	–	–	–	+
<i>B. japonica</i>	+	–	+	–	+
<i>B. tanukii</i>	–	–	+	–	+
<i>B. turdi</i>	–	+	–	–	+
<i>B. andersonii</i>	–	+	–	–	+
<i>B. bissettii</i>	–	+	–	–	+

* With the exception of one strain (GenBank accession no. D67020).

nymphs were opened at the posterior part of their opisthosoma using a razor blade or needle and fixed with 3·7% formaldehyde in PBS for at least 12 h at 4 °C. Embedding in cold polymerizing resin (polymerizing temperature 11 °C; Technovit 8100, Heraeus Kulzer) was performed according to the manufacturer's instructions. Polymerized resin blocks were stored at 4 °C. Semi-thin tick sections (4 µm) were obtained with a steel knife with hard metal edges on a rotary microtome (type 0036DDM; Medim), floated on sterile water, collected on silanized (3-aminopropyltrimethoxysilane; Sigma-Aldrich) microscope slides (Menzel-Gläser) and air-dried. Sections were stained with haematoxylin/eosin to correlate the histology with structures recognized by auto-fluorescence. *In situ* hybridization was carried out as described previously (Moter *et al.*, 1998) with slight modifications: hybridization buffer contained 20% (v/v) formamide, and slides were rinsed only once with sterile double-distilled water after hybridization. In each experiment, fixed cells of cultured spirochaetal strains as well as *Borrelia*-negative tick sections were included as controls. At least three sections per tick were analysed. Hybridized sections were examined with an epifluorescence microscope (Zeiss Axioskop) equipped with a 50 W high-pressure mercury lamp (HBO50; Osram) at ×100, ×400 and ×1000 magnification. Narrow-band filter sets HQ-F41-007 and HQ-F41-001 (AHF) were used to observe fluorescein and FluoX, and Cy3 signals, respectively. Photomicrographs were taken on Kodak Ektachrome HC400 films. Haematoxylin/eosin-stained sections were analysed under light using the same microscope.

EM. Two AZ/10-infected *I. ricinus* ticks were processed for EM to confirm the spirochaetal infection in the tick batch used. Briefly, the ticks were cut longitudinally using a razor blade under ice-cold fixative [3·5% glutaraldehyde in Sørensen phosphate buffer (0·1 M KH₂PO₄, 0·1 M Na₂HPO₄, pH 7·4)]. After 2 h, the fixative was diluted by adding Sørensen phosphate buffer (buffer/fixative 4:1) and stored at 4 °C until the fixation process was completed. Over a period of 2 h the specimens were rinsed several times with buffer solution and post-fixed with 2% buffered OsO₄ solution. After a further 2 h, the specimens were again rinsed with the buffer (three times for 10 min each) and were then dehydrated using a graded ethanol series and transferred to Araldite using propyleneoxide as an intermedium. Polymerization occurred at 65 °C. Sectioning was performed with a Leica ultracut UCT (Wetzlar) using a diamond knife. Ultrathin sections were

transferred to Formvar-covered copper grids. Sections were double-stained with uranyl acetate and lead citrate according to Reynolds (1963). EM-prepared tick sections were examined with a Zeiss EM 10CR transmission electron microscope.

RESULTS

PCR and dot-blot hybridization

The lower detection limit of the genus-specific amplification protocol was about 5–10 micro-organisms. Using an artificial DNA mixture containing rare templates (1 pg) from different *Borrelia* genospecies, the targets were detected even in the presence of a 100-fold excess of templates representing other genospecies. However, in cases of only weak amplicons, reamplification was necessary (data not shown) to increase the signal intensity in the subsequent analysis: in dot-blot hybridization experiments, LB-group-specific oligo-

Fig. 1. Dot-blot hybridization of a membrane using LB-group-specific oligonucleotide probes labelled with digoxigenin. DNA of four *Borrelia* control strains, *B. burgdorferi* ss B31^T (A), *B. afzelii* PKo (B), *B. valaisiana* VS116^T (C) and *B. garinii* A (D), was PCR-amplified using the genus-specific primer set Borr0/Borr4 and transferred to the membrane. Lanes 1–4 were then probed with BbV1, BaV1, BV and BgV1, respectively.

Table 3. PCR analysis of unfed *I. ricinus* nymphs which took their larval blood meal on gerbils *in vivo*-infected with *B. afzelii* and *B. burgdorferi* ss

Infection trial*	PCR-positive	Dot-blot hybridization		Mixed infection
		BaV1-positive	BbV1-positive	
A	12	5	10	3
B	17	17	0	0

* A, three gerbils first fed on by *B. afzelii*-infected *I. ricinus* nymphs and 27 d later by *B. burgdorferi* ss-infected *I. scapularis* nymphs; B, three gerbils first fed on by *B. burgdorferi* ss-infected *I. scapularis* nymphs and 45 d later by *B. afzelii*-infected *I. ricinus* nymphs. Eighteen ticks were tested in both trials.

Table 4. Specificity of oligonucleotide probes assessed by FISH

Spirochaetal strain	reBaV1	reBbV1	reBgV1	reBV	Borr4	EUB338
<i>B. afzelii</i> PKo	+	—	—	—	+	+
<i>B. burgdorferi</i> ss B31 ^T	—	+	—	—	+	+
<i>B. garinii</i> A	—	—	+	—	+	+
<i>B. valaisiana</i> VS116 ^T	—	—	—	+	+	+
<i>B. hermsii</i> HS1	—	+	—	—	+	+
<i>T. denticola</i> ATCC 33521	—	—	—	—	—	+

Fig. 2. FISH on an artificial mixture of *B. afzelii* (PKo) and *B. burgdorferi* ss (B31^T) using two probes simultaneously: reBaV1-Cy3 (red) and reBbV1-fluorescein (green). (a) *B. afzelii*, (b) *B. burgdorferi* ss. Bar, 20 μ m.

nucleotide probes were used to identify the respective amplicons (Fig. 1).

Mixed infection in ticks

Two series of infections were performed to produce gerbils double-infected with *B. afzelii* and *B. burgdorferi* ss. Putatively mixed-infected xenodiagnostic ticks

showed an *Borrelia* infection rate of 100% as analysed by IFA. The resultant unfed nymphs were analysed by PCR and dot-blot hybridization at least 5 months after post-larval repletion; however double-infected ticks were rarely found (Table 3). Interestingly, in most cases the genospecies which was used in the second of two infection steps on the gerbil was detected in the xenodiagnostic ticks. The apparent rarity of mixed

infected nymphs in our experiments prevented the planned inclusion of some of them in the FISH study.

FISH for direct visualization of borreliae

Oligonucleotide probes Borr4 and EUB338, complementary to semi-conserved and conserved regions of the 16S rRNA, respectively, as well as probes specific within the group of LB-associated genospecies, reBaV1, reBbV1, reBgV1 and reBV, reacted with the appropriate control strains (Table 4, a representative example in Fig. 2a, b).

A strong and bleaching-stable orange-yellow signal was observed in tick sections using the two Cy3 fluorescence-labelled probes, Borr4 and reBaV1. *B. afzelii* cells mainly clustered conspicuously adjacent to midgut epithelial cells (Fig. 3b–e). The typical spirochaetal morphotype (Fig. 4) was found in five out of six putatively *B. afzelii*-positive *I. ricinus* nymphs. Borreliae could not be detected in the salivary glands nor in other tick tissues, for example muscles or the central ganglion. Multiple target labelling by simultaneous use of Borr4/Cy3 and reBaV1/Cy3 did not result in an enhancement of the fluorescence signal. Due to the low signal-to-noise ratio, all attempts to visualize borreliae in ticks using fluorescein- or FluoX-labelled oligonucleotide probes failed (data not shown). However, autofluorescence of certain tick tissue structures allowed good histological orientation in the gross tick anatomy (Fig. 3a). Spirochaetes were not detected in any of the *Borrelia*-negative ticks.

As compared to tick tissues, the autofluorescence intensity was much lower in the gerbil skin sections, resulting in an excellent visualization of single spirochaetes, even with the FluoX-labelled oligonucleotide probe EUB338 (Fig. 5a, b). Borreliae were found mainly in the intercellular space of the lower fat cell layer close to subcutaneous muscles. Only a few borreliae were detected in biopsy sections (8 out of 22) taken directly from the tick attachment site. In contrast, all skin sections analysed ($n = 6$) taken 1.5 cm away from the tick-bite site – originally designed as negative controls – contained 5–15 single spirochaetal cells per section situated adjacent to the muscle cell layer, suggesting a rather fast migration of the borreliae within the fat cell layer towards deeper parts of the skin.

Ultrastructural analysis

To confirm the results of the FISH on tick whole-body sections, EM was performed on individual *I. ricinus* nymphs derived from the identical tick batch. The ultrastructural analysis revealed that the *I. ricinus* batch used was highly infected. *B. afzelii* cells were found in high numbers intracellularly in the midgut epithelium but also in the midgut lumen (Fig. 6). Infection of salivary glands could not be demonstrated. Spirochaetes were usually cross-sectioned; longitudinally sectioned cells exhibiting the typical spiral morphotype only rarely occurred.

DISCUSSION

An easy way to detect borreliae in ticks is by either dark-field microscopy (e.g. Burgdorfer *et al.*, 1982; Piesman *et al.*, 1991) or direct immunofluorescence assay on crushed tick samples (Kahl *et al.*, 1998b). Both methods are reliable, easy to perform in large series, and give good semi-quantitative results. Given the higher sensitivity and the possibility of storing the slides for several months, the direct or IFA can be considered as the method of choice to determine *Borrelia* infection in ticks. In principle, it allows the specific detection of *Borrelia* genospecies and detection of outer-surface protein expression when appropriate monoclonal antibodies are applied (Wilske *et al.*, 1993, 1995; Fingerle *et al.*, 1995; Masuzawa *et al.*, 1996).

However, more sophisticated techniques are required to investigate the spatial distribution of borreliae within the tick. Furthermore, the *in toto* preparation of tick organs, for example salivary glands and midgut, without cross-contamination (Piesman, 1995) is not only extremely time-consuming but also requires careful controls. In the past, the distribution of *B. burgdorferi* sl within *Ixodes* vectors has been studied by several authors: by light microscopy using either Giemsa stain (Burgdorfer *et al.*, 1982), silver dyes (e.g. Benach *et al.*, 1987; Lebet & Gern, 1994) or fluorescence-labelled antibodies (e.g. Piesman *et al.*, 1986; de Silva & Fikrig, 1995; Coleman *et al.*, 1997; Leuba-Garcia *et al.*, 1998), and also by EM (Burgdorfer *et al.*, 1989; Zung *et al.*, 1989; Zhu, 1998a, b). Although high magnification in the latter method allows resolution of most cellular details, for example the epithelial cells of the Malpighian tubules, EM is not suited for large-scale series. Furthermore, bacteria such as *Borrelia* genospecies cannot be differentiated by morphological criteria. EM seems to be more sensitive when compared to FISH. This could be due to the fact that EM mainly shows cross-sectioned spirochaetes which were seen in sections applied to FISH as well, but they cannot be identified with certainty even when showing a strong fluorescence signal (arrow in Fig. 4). On the other hand, in salivary glands no borreliae could be detected using FISH; unfortunately in this respect FISH could not be confirmed by EM, because in EM no salivary glands could be shown. They were probably lost during fixation of the ticks which were divided into two halves for EM embedding. However, assuming that a sufficient number of samples and sections are analysed, FISH seems to be well suited to study, for example, the distinct infection rate of the salivary glands in *I. ricinus* versus *I. scapularis*. Depending on the specific purpose of a particular study, each of the above-mentioned methods can be applied to detect spirochaetes in parts of a particular tick organ, or in relation to connective tissues when sections are prepared.

The purpose of the present study was to visualize borreliae in longitudinally *in toto*-sectioned *I. ricinus* ticks and to analyse the spatial distribution of the spirochaetes within different tick tissues. Demonstration

Fig. 3. For legend see page 1433.

Fig. 3. For legend see facing page.

Fig. 3. Detection of borreliae by FISH in whole-body sections of an *I. ricinus*-nymph (2.5 months post-ecdysis), infected with *B. afzelii* AZ/10 during the larval blood meal. FISH was carried out using the *Bacteria*-specific probe EUB338-fluorescein and the *Borrelia* genus-specific probe Borr4-Cy3 (b–d) or the *B. afzelii*-specific probe reBaV1-Cy3 (e). (a) Autofluorescence of a longitudinal-horizontally sectioned whole *I. ricinus* nymph. Note the opened cuticle at the posterior end for the entry of the fixative and embedding solutions. Tissue autofluorescence reveals the histology of the tick. P, palps; H, hypostome; CG, central ganglion; SG, salivary glands; M, pre-transversal muscles; PM, postero-medial muscles; MG, midgut; C, cuticle; SP, spiracular plate. (b) Posterio-lateral part of the tick section (see inset). Parts of the midgut are apparently separated by cross-sectioned pre-transversal muscles (at the upper border of the picture). The distinct orange-yellow line (arrowhead) within the midgut indicates a cluster of borreliae. (c) Detail of (b) showing a high resolution micrograph of the midgut borreliae. (d) Large numbers of intercellularly located borreliae clustering adjacent to the midgut epithelial cells. (e) Section of an AZ/10-infected *I. ricinus* nymph. Identification of *B. afzelii* within the tick midgut using the *B. afzelii*-specific probe reBaV1-Cy3. Bars: (a) 100 μ m, (b) 20 μ m, (c–e) 5 μ m.

Fig. 4. Section of the same tick individual as in Fig. 3(a–d). The probes Borr4-Cy3 and EUB338-fluorescein were used. Clear visualization of the spirochaetal morphotype in one optical section within the tick midgut. Strong fluorescence signal of cross-sectioned borreliae (arrowhead) does not allow the unequivocal detection of spirochaetes. Bar, 5 μ m.

Fig. 5. *Borrelia* cell in a gerbil skin punch biopsy. FISH using the *Borrelia* genus-specific probe Borr4-Cy3 (a) and the bacteria phylotype-specific probe EUB-Fluox (b) showing a single spirochaete in the intercellular space between fat cells. Bar, 5 μ m.

Fig. 6. Ultrastructure of a *B. afzelii*-infected *I. ricinus* nymph (2.5 months post-ecdysis). Borreliae in the lumen of the midgut close to the microvillus border. B, borreliae; M, mitochondria; MV, microvilli; N, nucleolus. Bar, 0.5 μ m.

of the borrelial morphotype was excellent in FISH experiments since the cells were exclusively visualized lying within one optical section of the sample. FISH on resin-embedded sections is well suited for the simultaneous study of the presence, localization and identity of a borrelial infection within a tick. However, since this depends upon the proper entry of the fixative and embedding resin into the tick, not all organs may be visible in all sections. Inadequately embedded ticks or engorged ticks with insufficient penetration of embedding solutions consistently disintegrated during

sectioning. Due to the autofluorescence of certain tick tissue structures and a relatively poor green signal resulting in a low signal-to-noise ratio, visualization of borreliae using fluorescein- or FluoX-labelled probes (EUB338, Borr4) was not successful and further fluorescent dyes should be tested for their suitability in FISH experiments on tick sections. The advantage of the method described here is the excellent preservation of the tissue architecture, its high specificity, i.e. the discrimination between genospecies, in combination with the exact localization of *Borrelia* cells in the tick tissues. This could be of considerable interest in mixed-infected ticks, which we failed to produce by stepwise feeding of infectious ticks on gerbils. It is known from previous studies that the Mongolian gerbil effectively transmits both *B. afzelii* and *B. burgdorferi* ss to feeding *Ixodes* ticks (O. Kahl, unpublished). In view of the fact that European *I. ricinus* populations very often harbour three or even four different *Borrelia* genospecies, it is intriguing that only a minority of the unfed nymphs carry a mixed infection. (Rijpkema *et al.*, 1996; Kurtenbach *et al.*, 1998; Richter *et al.*, 1999; Humair *et al.*, 1999; Gray *et al.*, 2000). The question of which factors prohibit more frequent mixed *Borrelia* infections to occur in unfed nymphs deserves further study.

In conclusion, the combination of embedding tick samples in cold polymerizing resin and FISH using Cy3-labelled probes results in an excellent signal-to-noise ratio. The *Borrelia*-specific probes described here are

well suited to study borreliae in whole ticks and clinical specimens, for example on biopsies of patients with questionable LB as was reported by Stanek *et al.* (1990) on an endomyocardial biopsy specimen. Moreover, because of their good performance in PCR, the newly designed primers could be helpful in establishing a diagnostic assay for LB patients.

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