UNIVERSITÉ DE NEUCHÂTEL FACULTÉ DES SCIENCES

INSTITUT DE ZOOLOGIE

LYME BORRELIOSIS

CIRCULATION OF *BORRELIA BURGDORFERI SENSU LATO* BETWEEN ITS HOSTS AND ITS VECTOR, *IXODES RICINUS*

PAR

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THÈSE
(FORME RÉDUITE)

PRÉSENTÉE À LA FACULTÉ DES SCIENCES DE L'UNIVERSITÉ DE NEUCHÂTEL
POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

NEUCHÂTEL - 1998

IMPRIMATUR POUR LA THÈSE

Borréliose de Lyme: circulation de *Borrelia burgdorferi sensu lato* entre ses hôtes et son vecteur, *Ixodes ricinus*

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Neuchâtel, le 5 juillet 1998

Le doyen:

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Forme réduite de la thèse

La forme réduite de la thèse est constituée des articles suivants:

- Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland.
  Humair P.F., Péter O., Wallich R. & Gern L.
  Journal of Medical Entomology (1995) 32: 433-438

- An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes.
  Humair P.F., Postic D., Wallich R. & Gern L.
  Zentralblatt für Bakteriologie (1998) 287: 521-538

- Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland.
  Humair P.F. & Gern L.
  Acta Tropica (1998) 69: 213-227

- Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance.
  Humair P.F., Rais O. & Gern L.
  Parasitology (1999) 118: 33-42

Un exemplaire du texte original *in extenso* de la présente thèse est déposé à la bibliothèque centrale de l'Université de Neuchâtel.

Strain Variation of Lyme Disease Spirochetes Isolated from *Ixodes ricinus* Ticks and Rodents Collected in Two Endemic Areas in Switzerland

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J. Med. Entomol. 32(4): 433–438 (1995)

ABSTRACT The relationship among Lyme borreliosis, *Borrelia* genospecies, rodent reservoirs, and *Ixodes ricinus* L. ticks was studied in two endemic areas in Switzerland. Ear punch biopsies and sampling of internal organs were used to isolate *Borrelia burgdorferi* (Johnson, Schmid, Hyde, Steigerwalt and Brenner) from small mammals, *Apodemus sylvaticus* L., *A. flavicollis* Melchior, *Clethrionomys glareolus* Schreber. Spirochetes were isolated from ear tissue and spleen of the rodents. Isolates were homogeneous and belonged to typing group II identified as *B. afzelii* (Canica, Nato, du Merle, Mazie, Baranton and Postic). Our data show that a specific association exists between *B. afzelii* and rodent reservoirs in European foci. Borreliae were also isolated from field-collected *I. ricinus* ticks from the same study areas. Proteinic and antigenic analysis indicated that more than one genospecies were present in the tick population. This suggests that other vertebrate hosts may serve as reservoirs of other *Borrelia* genospecies implicated with Lyme disease.

KEY WORDS Lyme borreliosis, *Borrelia afzelii*, rodents

Borrelia burgdorferi Johnson, Schmid, Hyde, Steigerwalt and Brenner, the causative agent of Lyme borreliosis, is maintained in nature by tick-vertebrate transmission. In European endemic foci, the three-host tick *Ixodes ricinus* L. is involved in the maintenance cycle, transmitting the spirochetes to animal hosts that serve as sources for infecting other ticks. Larvae mainly feed on small mammals, whereas nymphs are more frequently found on birds and medium-sized mammals (Aeschlimann 1972). In Switzerland, the prevalence of infected questing larval ticks is low (3.1%) (Miserez et al. 1990, Zhioua et al. 1994), whereas the infection rates of *B. burgdorferi* in host-seeking nymphs and adults range from 10 to 55% (Aeschlimann et al. 1986, Miserez et al. 1990, Péter 1990, Lebet and Gern 1994, Leuba-Garcia et al. 1994). Rodents including *Apodemus flavicollis* Melchior, *A. sylvaticus* L., and *Clethrionomys glareolus* Schreber were shown to be reservoir hosts because they may remain infective for ticks for a long time after the primary infection (Aeschlimann et al. 1986, Humair et al. 1993a, Gern et al. 1994b).

Lyme spirochetes have shown a great diversity concerning their pheno- and genotypic characters (Barbour et al. 1985, Wilske et al. 1988, Kramer et al. 1990, Péter and Bretz 1992, Wallich et al. 1992). Using various methods, three genospecies have now been described in Europe: *B. burgdorferi sensu stricto*, *B. garinii* Baranton, Postic, Saint Girons, Boerlin, Piffaretti, Assous and Grimont, and *B. afzelii* Canica, Nato, du Merle, Mazie, Baranton and Postic (Baranton et al. 1992, Canica et al. 1993). These genomic species can be routinely identified by monoclonal antibodies (Canica et al. 1993). Besides, recent studies have demonstrated that various phenotypes of *B. burgdorferi* are present in the tick population of one endemic area (Péter and Bretz 1992, Boerlin et al. 1992) and that their distribution greatly differ from one area to another (Hu et al. 1994a). The reason for such a diversity in a focus remains unknown but could be caused by the presence and frequency of various reservoir hosts.

In this study, we were interested in isolating spirochetes from rodents and from host-seeking ticks collected in two endemic areas to compare their phenotypic characteristics.

Materials and Methods

Study Sites. Collection of rodents and ticks were conducted in two endemic woodlands in Switzerland: Glütschbachtal near Thun (site A) (Canton of Berne, altitude 589 m) and a forest close to Martigny (site B) (Canton of Valais, altitude 484–500 m).

Investigation of Rodents. Small mammals were live-trapped at both study sites from May to October 1993. Traps baited with cereal granules, sunflower grains, and apples were set once monthly in

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0022-2585/95/0433–0438$02.00/0 © 1995 Entomological Society of America

the evening and checked the two following mornings. Captured rodents were brought to the laboratory and caged individually. Animals were identified to species on the basis of morphometric measurements and pelage coloration, identified by sex, and weighed.

Animals from site A were anesthetized, marked, and an ear punch biopsy was performed for isolation of *B. burgdorferi*. Rodents were finally released at the site where they were captured. Animals from site B were killed by carbon dioxide inhalation in an euthanasia chamber and dissected. Cultures were prepared from samples of bladder, spleen, heart, brain, cheek skin, and ear.

Animal care and manipulations were in accordance with the Swiss Federal Animal Welfare Laws (LPA and OPA). This study was conducted in accordance with the permits issued by the Swiss Federal Forest Department and the Cantonal Department of Agriculture.

Collections of Ticks. Nymphal and adult *I. ricinus* ticks were collected from vegetation by flagging. One-hour flagging was done once a month at Glütschbachtal, during August–October 1993. Ticks collected were identified to species, stage, and sex.

Isolation of Spirochetes. Ear samples were removed from anesthetized animals after cleaning the ear with 70% ethanol. Ear punch biopsies and dissected tissues from bladder, cheek skin, heart, spleen, and brain were placed into tubes containing supplemented Barbour Stoenner Kelly (BSKII) medium as described by Sinsky and Piesman (1989).

Field-collected ticks were briefly soaked in 70% ethanol before they were individually squashed with sterilized forceps in tubes containing BSKII medium (Barbour 1984) supplemented with 50 μg/ml Rimactan (Ciba, Basel, Switzerland) and 50 μg/ml Fosfocin (Boehringer, Mannheim, Germany).

Dark-field microscopy was used to screen inoculated cultures for the presence of spirochetes after 10 d, and 3, 6, and 8 wk of incubation at 34°C. Positive cultures were inoculated into fresh tubes of BSKII medium (Barbour 1984) for additionnal growth and evaluation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and Southern blot.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Electrophoresis and immunoblots were performed as previously described (Péter and Bretz 1992). In short, the suspension of washed borreliae (1 mg/ml) were dissolved (1:1) in the sample buffer with 0.6% sodium dodecyl sulfate (SDS) (final concentration) and 50 mM dithiothreitol as a reducing agent. The samples were boiled for 5 min before undergoing electrophoresis (constant current 45 mA) on polyacrylamide gel at 12.5% for the separating gel and a 4% stacking gel, according to the method described by Barbour et al. (1985). The standard molecular weights (MW) of BioRad (low range protein molecular weight standards) were used as reference for the calculation of relative molecular weights. After electrophoresis, proteins were transferred by Western blot to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Kloten, Switzerland) in accordance with the method of Towbin et al. (1979).

Immunoblotting and Typing of Isolates. After transfer, the membrane was stained with Coomassie blue. The membrane was then cut at the level of the outer surface proteins OspA and OspB as well as below 14.4-kDa marker, and these two pieces were destained in a bath of pure methanol for a few seconds. They were saturated with 3% gelatine in a Tris buffer of pH 7.5 for 1 h at 37°C and washed three times for 5 min in a Tris-Tween 20 (0.05%) buffer. The piece containing the OspA and OspB were incubated for 2 h at room temperature with monoclonal antibodies (MAbs) H3TS and I 17.3 diluted 1:500 and 1:500 000, respectively, in the same buffer with additional 1% gelatine. The piece below 14.4 kDa was incubated as above with monoclonal antibody D6 diluted 1:100. After washing, MAbs fixed specifically on the antigens were demonstrated by a second goat anti-mouse IgG for H3TS and I 17.3 MAb or goat anti-mouse IgM for D6 MAb conjugated to alcaline phosphatase, followed by three washes and the addition of BCIP/NBT substrate (5 bromo-4-chloro-3-indoyl p-toluidine phosphate/p-nitro blue tetrazolium chloride).

Southern Blot Hybridization. Total genomic DNA was extracted from *Borrelia* organisms as described previously (Wallich et al. 1992). Briefly, about 5 μg of DNA was digested with 100 units of restriction nuclease *Hind*III according to manufacturer recommendations (Boehringer). Samples were electrophorized using a 0.7% agarose gel. DNA fragments were transferred to Hybond-N nylon membrane (Amersham Buchler, Braunschweig, Germany) followed by UV-cross-linking and hybridization as described (Wallich et al. 1992). Shortly, using ^{32}P-labeled *ospA* gene probe, hybridization was performed overnight at 65°C in 0.5 M $NaHPO_4$/7% $NaDodSO_4$, 7.2 pH. After washing in 40 mM $NaHPO_4$/1% $NaDodSO_4$, 7.2 pH at room temperature for 30 min, the dry membrane was autoradiographed on Kodak XAR-5 film with intensifying screens at −80°C for 1–12 h.

Results

Rodent Isolates. In total, 111 small mammals were captured from May through October 1993 at Glütschbachtal near Thun (site A): 60 *C. glareolus*, 22 *A. flavicollis*, 21 *A. sylvaticus*, 2 *Apodemus* sp., and 6 *Sorex araneus* L. In Martigny (site B), 41 small mammals were captured: 31 *A. flavicollis*, 9 *A. sylvaticus*, and 1 *Glis glis* L.

In site A, ear punch biopsies were removed from 55 *C. glareolus*, 22 *A. flavicollis*, 21 *A. sylvaticus*, and 2 *Apodemus* sp. Fourteen isolates of borreliae

SITE A

MW (kDa) | Rodent isolates | Tick isolates

94 — 66 — 43 — 31 — 21.5— 14.4—

	NE 28	NE 29	NE 30	NE 36	NE 45	NE 37	NE 38	NE 41	NE 39	NE 40	NE 42	NE 43	NE 44	NE 35	NE 47	NE 48	NE 49	NE 50	NE 51	NE 52	NE 53
Reactivity with mab																					
H3TS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
I17.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	(+)	-	-	-	-	+
D6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
Southern blot OspA Hind III fr. kb	1.7	1.7	1.7	1.7	n.d.	1.7	1.7	1.7	1.7	1.7	n.d.	1.7	1.7	1.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Fig. 1. Protein profiles of *Borrelia* isolates from rodents and ticks from site A. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi sensu stricto*), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). Southern blotting: size (kb) of *Hind*III fragment for the *ospA* gene. MW, molecular weight standard; NE28-NE30 and NE36-NE45, isolates from *C. glareolus* voles; NE35, isolate from *A. flavicollis* mouse; NE47-NE49, isolates from *I. ricinus* females; NE50-NE52, isolates from *I. ricinus* males; NE53, isolate from *I. ricinus* nymph. +, positive reaction; (+), lightly positive reaction; −, negative reaction; n.d., not done.

were cultivated from ear tissue biopsies of 10 *C. glareolus* (NE28-30 and NE36-45) and 1 *A. flavicollis* (NE35) (Fig. 1). Three isolates (NE30, NE36, and NE45) were obtained from one *C. glareolus* captured in June (NE30), recaptured in July (NE36), and maintained in the laboratory until October (NE45). Two isolates (NE38 and NE41) were obtained from another vole trapped in July (NE38) and retrapped in August (NE41).

In site B, two isolates of borreliae were cultivated, one from the ear biopsy of one *A. flavicollis* (VS 25R) and the other from the spleen tissue of one *A. sylvaticus* (VS 42R) (Fig. 2).

Protein profiles (Figs. 1 and 2) showed a great homogeneity. All rodent isolates possessed an OspA of 32 kDa and an OspB of 35 kDa. A major band of 23 kDa was generally visible, except in two cases (Fig. 1: lane 6, NE37 and Fig. 2: lane 2, VS 42R). By Western blotting, all rodent isolates reacted with MAb I 17.3 which recognizes *B. afzelii*, but did not react with MAb H3TS which specifically reacts with *B. burgdorferi sensu stricto* or MAb D6 which identifies *B. garinii* isolates (Figs. 1 and 2).

Southern blotting confirmed the homogeneity of the rodent isolates from site A (Fig. 1). All showed a single DNA fragment of 1.7 kb for the *ospA* gene as the strain ACA1 (Wallich et al. 1992).

Tick Isolates. The cultivation of 40 *I. ricinus* (22 nymphs, 11 females, and 7 males) from site A yielded seven isolates, three from females (NE47-49), three from males (NE50-52), and one from a nymph (NE53). From site B, six isolates were obtained from 55 adults, three from females (VS 134, 137, and 641), and three from males (VS 14, 619, and 623).

Protein profiles of tick isolates (Fig. 1) from site A clearly showed a heterogeneous pattern. The OspA, varying between 31 and 33 kDa, was always present, whereas the OspB (33–35 kDa) was not expressed in two isolates (lanes 19 and 20, respectively NE51 and NE52). The 21.5–24 kDa proteins of tick isolates presented a bigger variability than that of rodent isolates.

Using Western blotting, two tick isolates—one from a female (NE48) and one from a nymph (NE53)—reacted with MAb I 17.3, which recognizes *B. afzelii* (Fig. 1). Three tick isolates (NE47, NE51, and NE52) reacted with MAb D6, which specifically identifies *B. garinii*. Reactivity with MAb H3TS, which recognizes *B. burgdorferi sensu stricto*, occurred three times among tick isolates: two from females (NE48 and NE49) and one from a male (NE50). Hence, NE48 reacted with both H3TS and I 17.3, suggesting that both genospecies were present in this female tick. Immunoblot of

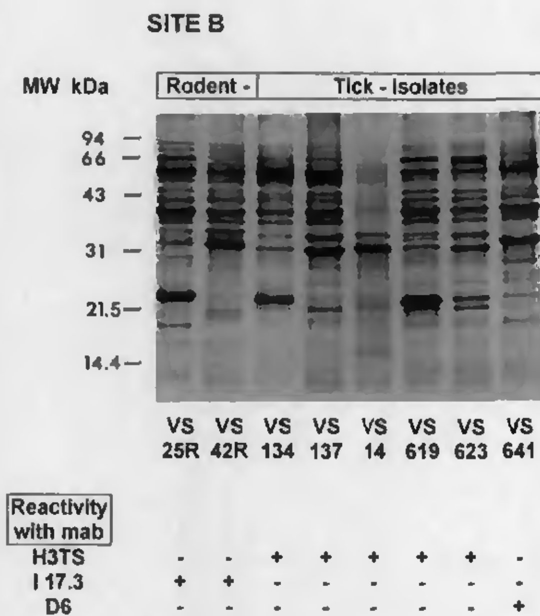

Fig. 2. Protein profiles of *Borrelia* isolates from rodents and ticks from site B. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi sensu stricto*), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). MW, molecular weight standard; VS 25R, isolate from *A. flavicollis* mouse; VS 42R, isolate from *A. sylvaticus* mouse; VS 134, VS 137, and VS 641, isolates from *I. ricinus* females; VS 14, VS 619, and VS 623, isolates from *I. ricinus* males.

NE48 isolate from a previous subculture (data not shown) showed a stronger reactivity with I 17.3 than with H3TS (immunoreactivity reversed in comparison to Fig. 1, NE48), suggesting that competition between strains may occur during cultivation.

Five of the isolates from site B showed protein profiles (Fig. 2) with an OspA of 31 kDa and an OspB of 34 kDa. The remaining isolate had an OspA of 32 kDa but no OspB (Fig. 2). Immunoblotting with MAb H3TS confirmed that the five isolates were *B. burgdorferi sensu stricto* and the other one reacted with MAb D6, specific for *B. garinii* isolates. None reacted with MAb I 17.3.

Discussion

This study was prompted by the results of a previous investigation showing that various *B. burgdorferi* strains may be present in an endemic area (Boerlin et al. 1992, Péter and Bretz 1992) and that some phenotypes may be more frequent in some foci than in others (Hu et al. 1994a). In nature, *B. burgdorferi* is maintained by tick-vertebrate transmission. Spirochetes present in ticks originate from hosts on which the ticks fed during their previous blood meal. Because rodents are competent amplifying hosts capable of transmitting spirochetes to a great number of larvae (Aeschlimann et al. 1986, Kurtenbach et al. 1992, Humair et al. 1993a, Gern et al. 1994b), we were interested in the phenogenotypes of spirochetes infecting small mammals.

In Europe, a great heterogeneity in the expression of the main outer surface proteins (Osp) of *B. burgdorferi* is present among tick and patient isolates. However, little is known about the quality of the spirochetes infecting wild mammals; only two isolations from rodents have been described so far (Hovmark et al. 1988). In the current study, a total of 16 isolates was obtained from ear punch biopsies or spleen of *Apodemus* mice and *Clethrionomys* voles. All were homogeneous and belonged to typing group II (Péter and Bretz 1992), which is now referred to as *B. afzelii* (Canica et al. 1993). Interestingly, the rodent isolate (SmS1) obtained by Hovmark et al. (1988) belongs to *B. afzelii* (Postic et al. 1994) as well as two strains isolated from rodents in Northern Italy (Genchi et al. 1994).

The first description of the ear punch biopsy method for isolation of *B. burgdorferi* showed that this method is appropriate for isolation of *B. burgdorferi sensu stricto* from hamsters and *Peromyscus leucopus* Rafinesque (Sinsky and Piesman 1989). Isolates obtained from ear punch biopsies of laboratory mice infected by the European strain ZS7 (Gern et al. 1994a), which was identified as *B. burgdorferi sensu stricto* (Wallich et al. 1992), confirm that no selection process caused by the medium is responsible for such an homogeneity among our rodent isolates.

However, recent studies (Gorelova et al. 1994, Khanakah et al. 1994) have shown that the different genospecies of the causative agent of Lyme disease were isolated by culture or detected by polymerase chain reaction in the internal organs of small mammals (urinary bladder, heart, spleen). Thus, a single rodent may be infected by more than one genospecies (Khanakah et al. 1994). The results of the current study reveal that only one genospecies, *B. afzelii*, is prominent in the ears of the rodents. This suggests that *B. afzelii* would demonstrate preferentially a cutaneous location in rodents, as observed in human skin lesions (Canica et al. 1993). This fact is epizootiologically important, because ticks feeding on rodents attach primarily to the ears (Arthur 1965). Thus, one would assume that ticks that had fed on infected rodents would harbor *B. afzelii*. This was confirmed by Hu et al. (1994b) who observed that isolates obtained from ticks that had been allowed to feed on infected *Apodemus* sp. mice were *B. afzelii*. Additionally, isolates from rodent-feeding *Ixodes persulcatus* Schulze in Japan were identified as *B. afzelii* (Nakao et al. 1994). Finally, even if rodents may be infected by a different genospecies of *B. burgdorferi*, our data coupled with those of Hu et al. (1994b), strongly suggest that a specific association exists between the type strain and *Apodemus* mice and *Clethrionomys* voles that appear to be the main reservoirs for the genospecies *B. afzelii*.

Isolates from host-seeking ticks were heterogeneous: the three genospecies were represented. This confirms the results observed in previous studies, showing a great diversity among *B. burgdorferi* strains isolated from ticks from endemic foci (Hu et al. 1994a; O.P., unpublished data). One of the female ticks collected in Thun was infected by more than one phenotype. This is in accordance with previous observations showing that adults may harbor more than one genospecies (Leuba-Garcia et al. 1994).

The heterogeneity of tick isolates contrasts the homogeneity of isolates from rodents. Our data indicate an association in nature between the genospecies *B. afzelii* and rodents, and suggest that other genospecies may be associated with other vertebrate hosts. Ground-foraging birds (e.g., thrushes, blackbirds, and robins) have been incriminated as potential reservoirs for the Lyme disease spirochete (Humair et al. 1993b) and could be associated with *B. garinii*. The recent observations of *B. garinii* in bird-feeding ticks (Nakao et al. 1994, Olsen et al. 1994) support such a hypothesis.

Further studies are needed to investigate the role of other potential hosts to understand the relationship between *Borrelia* genospecies and vertebrate reservoirs.

Acknowledgments

We thank Christiane Brenner, Anne-Gabrielle Bretz, Mafalda Vieira da Silva, and Olivier Rais for their excellent technical assistance, and Alan Barbour and Guy Baranton for the monoclonal antibodies. This research was supported by the Swiss National Science Foundation (No. 32-29964-90) and the Swiss Federal Office of Education and Science (No. 93.0363). This work is part of the Ph.D. dissertation of P.F.H.

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Received for publication 19 July 1994; accepted 30 December 1994.

Zent.bl. Bakteriol. 287, 521–538 (1998)
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Zentralblatt für
Bakteriologie

An Avian Reservoir *(Turdus merula)* of the Lyme Borreliosis Spirochetes

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Received April 23, 1997 · Revision received July 22, 1997 · Accepted October 24, 1997

Summary

The reservoir competence of passerine birds for the Lyme borreliosis spirochetes was studied in an enzootic focus in Switzerland. Skin aspirates and skin biopsies were used to isolate *Borrelia* spirochetes from *Turdus* species. *B. burgdorferi* sensu lato was isolated and/or PCR-detected in BSK medium containing skin biopsy or skin aspirate from 5 blackbirds *(T. merula)* and one song thrush *(T. philomelos)*. Seven isolates were obtained from 3 different blackbirds. Either *B. garinii* or *Borrelia* from the genomic group VS116 was found in bird skin samples. Mixed infection occurred in 2 cases. Tick xenodiagnosis was used to determine whether blackbirds transmitted *Borrelia* to ticks. Five xenodiagnoses were performed on 3 different blackbirds. *Borrelia* DNA was detected in BSK medium inoculated with xenodiagnostic ticks from all the passerines tested. Isolates cultured from xenodiagnostic ticks were obtained from 2 blackbirds. Isolates belonged to group VS116 (n = 10) and to *B. garinii* (n = 1). Our study has shown that *Turdus* sp. are infected by *B. garinii* and by *Borrelia* from group VS116 and that blackbirds are implicated as reservoirs for these 2 genomic groups of *Borrelia,* as they transmit living borreliae to ticks. An association seems to exist between birds and *Borrelia* VS116, and to a lesser extent, *B. garinii,* similar to the association existing between small rodents and *B. afzelii.* Our observations emphasize the fact that different enzootic cycles maintain Lyme borreliosis spirochetes in nature.

Introduction

Lyme borreliosis is a infectious disease of humans caused by spirochetes of the *Borrelia burgdorferi* complex. In Europe, three genospecies, *B. burgdorferi* sensu stricto (ss), *B. garinii*, and *B. afzelii* (6, 8, 18) are implicated as causative agents of Lyme borreliosis in humans. Two other borrelial genomic groups, group VS116 and group PotiB2, have been described in Europe (29, 31) but appear to be non-pathogenic for humans. The three-host tick, *Ixodes ricinus*, is the main vector of *B. burgdorferi* sensu lato (sl) in European endemic areas. Lyme disease borreliae are maintained in nature by enzootic transmission cycles involving tick vectors and vertebrate reservoirs. Although *I. ricinus* parasitizes a wide range of hosts including reptiles, birds, and small, medium, and large-sized mammals (1, 5), ecological studies on Lyme borreliosis have mainly focused on mammals, and particularly on small rodents. The contribution of *Apodemus* mice and *Clethrionomys* voles as competent reservoirs in Europe has been described extensively (2, 9, 16, 19, 22, 35).

In contrast to small mammals, the involvement of birds in the ecology of Lyme borreliosis remains controversial. *B. burgdorferi* sl has been isolated from passerines and/or from ticks of the *I. ricinus* complex collected from various species of birds in Europe, North America and Japan (3, 4, 14, 17, 20, 24, 26, 27, 34). However, two experimental studies have shown that the American catbird *(Dumetella carolinensis)* and the European blackbird *(Turdus merula)* failed to transmit the spirochete to xenodiagnostic ticks (21, 23). Furthermore, *Matuschka* and *Spielman* (23) described blackbirds as having a zooprophylactic role: infected ticks lost their infection when feeding on two blackbirds. These findings are in contrast to those of field studies which have shown that infected ticks can be collected from indigenous birds (4, 14, 17, 20, 34) and from migratory species (25, 27, 37). Moreover, the existence of a transmission cycle involving seabirds and *I. uriae* (28) has shown the importance of avian hosts in the ecology of Lyme borreliosis. In view of such conflicting observations, the role of passerines in the transmission cycle of *B. burgdorferi* sl needs to be clarified.

In the present study, we focused on blackbirds and thrushes, passerines that were found to be frequently infested with *I. ricinus* ticks in a previous study of an area endemic for Lyme borreliosis (17). *B. burgdorferi* sl was isolated from birds captured at this field site and from field-derived and xenodiagnostic bird-feeding ticks. Phenotypic and genotypic characteristics of the isolates were compared.

Materials and Methods

Study site

Birds were collected at the "Staatswald" (Ins, Canton of Berne, Switzerland, altitude: 433 m), a woodland previously described as an enzootic focus of Lyme borreliosis (2, 12, 16, 17).

Examination of birds

Birds were captured in Japanese mist nets from 1 August through 15 September 1995. Ground-level nets were placed at sites adjacent to raspberry and blackberry bushes, a food source at that time of year for ground-frequenting passerines (blackbirds, thrushes, robins). Each captured bird was identified to species, marked by rectrice-clipping, and if possible, sexed and aged. Birds belonging to the target group (blackbirds, thrushes, robins) were brought to the laboratory where they were examined for ticks and samples were taken for spirochete isolation. They were released at the field site. Other birds were examined for ticks in the field and released at the capture site immediately after tick examination. All recaptured birds were reexamined for ticks. Ticks were placed into labelled vials and stored in humid chambers at room temperature.

Blackbirds were kept at our laboratory for a short period to enable tick xenodiagnosis, and then released at the capture site. Recaptured blackbirds were retested by xenodiagnosis.

Tick xenodiagnosis

Blackbirds were individually caged over a pan of water. Prior to xenodiagnosis, field-derived ticks feeding on blackbird No. 1 were collected engorged in the pan of water after drop-off. To reduce the time in captivity, field-derived ticks feeding on other blackbirds were removed by forceps. Xenodiagnosis was undertaken 1–3 days after all field-derived ticks had become detached. Xenodiagnostic *I. ricinus* larvae reared from a laboratory colony free of spirochetal infection were placed on the head of birds, and particularly on the skin around the bill. Replete ticks were collected daily in the pan of water and allowed to moult to the nymphal stage.

Collection of ticks

Free-living *I. ricinus* nymphs and adults were collected by flagging the vegetation at the site of bird mist netting during August and September 1995. Ticks were identified to species, stage and sex.

Isolation of spirochetes

Needle aspiration, skin biopsy and blood culture were used to isolate spirochetes from blackbird *(T. merula)*, song thrush *(T. philomelos)*, robin *(Erithacus rubecula)* as well as dunnock *(Prunella modularis)* and blackcap *(Sylvia atricapilla)*. Needle aspiration of skin as described by *Piesman* et al. (30) was adapted to passerines. Approximately 0.1 to 0.3 ml of BSKII was injected intradermally with a 1 ml syringe and 26-G needle into the thighs or chin. The fluid was simultaneously reaspirated with a second needle inserted near the site of inoculation. About 0.02 to 0.15 ml of fluid were recovered by needle aspiration. Skin samples ($\leq$ 1 mm^2) were removed from chin after cleaning the skin with 70% ethanol. Blood samples ($\leq$ 0.05 ml) were taken from the brachial vein with a 26-G needle and a 1 ml syringe. Aspirates, skin and blood samples were immediately inoculated into tubes containing BSKII medium supplemented according to *Sinsky* and *Piesman* (33).

Host-seeking, and moulted field-derived and xenodiagnostic bird-feeding ticks were briefly soaked in 70% ethanol and squashed with sterilized forceps in tubes containing BSKII medium as modified by *Sinsky* and *Piesman* (33).

Inoculated cultures were screened by dark-field microscopy for the presence of spirochetes after 10 days, and 3, 4, 6, and 8 weeks of incubation at 34 °C. Positive cultures were subcultured for analysis by SDS-PAGE, Western blot and Southern blot.

SDS-PAGE and Western blot

After 2–5 subcultures, bird and tick isolates were analyzed by SDS-PAGE and Western blotting. Suspensions of washed spirochetes (2 mg/ml) were dissolved (1 : 1) in sample buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol as a reducing agent and then subjected to polyacrylamide gel electrophoresis on 6% stacking gel and 12.5% separating gel. Protein bands were visualized with the aid of Coomassie brilliant blue R-250 staining. Low-range protein molecular weight standards (Bio-Rad) were used as references.

Following electrophoresis, proteins were transferred by Western blot onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blots were cut widthwise at the level of the 14.4 kDa marker and membrane-containing proteins above the 14.4 kDa marker were cut lengthwise into two strips. Membrane strips containing OspA and OspB were incubated overnight at room temperature with monoclonal antibodies (MAbs) H3TS and I 17.3 diluted 1 : 500 and 1 : 100 000, respectively. MAb H3TS recognizes the OspA (31 kDa) of *B. burgdorferi* ss (7), and I 17.3 reacts with the OspB (35 kDa) of *B. afzelii* (8). Membranes with a 14.4 kDa marker were incubated as above with MAb D6 diluted 1 : 50; this MAb identifies a 12 kDa protein of *B. garinii* (29). Nitrocellulose papers were then incubated with peroxidase-conjugated goat anti-mouse IgG for H3TS and I 17.3 MAbs, and goat anti-mouse IgM for D6 MAb (Nordic Immunological Laboratories, The Netherlands). Finally, membranes were immersed in substrate solution containing 4-chloro-1-naphtol and 30% hydrogen peroxide, washed in distilled water and air-dried.

RFLP analysis

The intergenic spacer between two duplicated ribosomal genes of *B. burgdorferi* sl was used as a template for the polymerase chain reaction (PCR) amplification and RFLP analysis, as described by *Postic* et al. (31). Briefly, 2–4 ml of initial culture were centrifuged and washed twice. The pellet was resuspended in 50 µl of sterile ultrafiltered water and boiled at 100 °C for 10 min. Ten µl of each sample were used as template for DNA amplification. Primer 1 (5'-CTGCGAGTTCGCGGGAGA-3') and primer 2 (5'-TCCTAGGCATTCACCATA-3') were used to amplify the variable intergenic spacer. The PCR amplification was carried out in 50 µl solution for 35 cycles in a thermal cycler (MJ Research, Watertown, MA, USA). Denaturation was performed at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. PCR products were electrophoresed in a 1% agarose gel and visualized by UV transillumination after ethidium bromide staining. For each PCR reaction, strains B31 (*B. burgdorferi* ss), NE632 (*B. afzelii*) and NE11H (*B. garinii*) were used as positive controls, and a number of water samples as negative controls. The usual precautions were taken to avoid cross-contamination. Amplified DNA was digested with *MseI* restriction endonuclease (New England Biolabs, Beverly, MA, USA) for 2 h at 37 °C. Digested DNA was electrophoresed in a 16% acrylamide gel for 1 h 30 min at 120 V, stained by ethidium bromide and visualized by UV transillumination. Marker V (Boehringer Mannheim, Mannheim, Germany) was used to estimate the size of DNA fragments.

Southern blot

Alternatively, total genomic DNA was extracted from the borreliae as previously described (36) and approximately 5 µg of DNA was digested with *Hind*III restriction endonuclease for 2 h at 37 °C. Digested DNA was separated by electrophoresis on a 0.7% agarose gel and was transferred to a Nytran Plus nylon membrane (Schleicher & Schuell, Keene, NH). Following UV cross-linking of the DNA, the membrane was dried and hybridized with ^{32}P-labelled probes. Gene probes were derived from *B. burgdorferi* ZS7 and included the chromosomal probes *fla, groEL, DnaK* and IpLA7. The membrane was autoradiographed on Kodak XAR safety film with intensifying screens at –70 °C for 20 min to 12 h.

Results

Investigation of birds

72 birds of 16 different species were captured in mist nets at the Staatswald woodland from August to September 1995: 10 *T. merula,* 1 *T. philomelos,* 16 *E. rubecula,* 1 *Troglodytes troglodytes,* 3 *P. modularis,* 18 *S. atricapilla,* 2 *S. borin,* 5 *Phylloscopus collybita,* 1 *Regulus regulus,* 1 *Ficedula hypoleuca,* 2 *Parus ater,* 1 *P. major,* 1 *P. montanus,* 8 *P. palustris,* 1 *Certhia familiaris* and 1 *Fringilla coelebs.* Six birds were recaptured once and 2 were recaptured twice.

Isolation and PCR detection of B. burgdorferi sl in cultures of bird tissues

From 27 various passerines, a total of 42 skin aspirates, skin biopsies, and blood samples was inoculated into BSKII for the isolation of spirochetes (Table 1). Seven *Borrelia* isolates were obtained from 3 different blackbirds (Table 2). RFLP of three isolates from bird No. 3 showed that a mixed infection with *B. garinii* and spirochetes of group VS116 was present in the skin of this bird (Table 2 and Fig. 1). When tested by SDS electrophoresis and Western blotting, these isolates showed an OspA of 33 kDa without OspB, and reacted with MAb D6, specific for *B. garinii* (Fig. 2). The mixed infection with spirochetes of group VS116 was not detected in protein profiles. The four other isolates were obtained from birds No. 4' and 5 and were identified as *Borrelia* VS116 by RFLP (Table 2 and Fig. 1). They expressed an OspA of 33.5 kDa and did not react with any of the MAbs used, as previously described for group VS116 (29). Among these 7 avian isolates, OspA of *B. garinii* and VS116 did not show any variability. OspC protein was either expressed (NE216, NE217, NE219), slightly expressed (NE218, NE223, NE225) or absent (NE224) (Fig. 2).

Using Southern blot, we observed that typical *B. garinii* isolates exhibited fragments at 1.2 & 0.5, 1.1 & 0.9, 0.6 & 0.7, and 0.6 kbp with the *fla, groEL, DnaK* and lpLA7 gene probes, respectively. In contrast, isolates belonging to the VS116 genogroup exhibited fragments at 1.5, 1.1 and at 0.9, 0.8, and 1.9 kbp, respectively.

Table 1. Detection of *Borrelia* DNA in BSK medium containing skin aspirate/biopsy or blood from passerine birds captured at the Staatswald forest (Ins, Switzerland, August–September 1995)

Common name	Scientific name	No. birds examined	Skin aspirate (thighs)[a]	Skin aspirate (chin)[a]	Skin biopsy (chin)[a]	Blood[a]
Blackbird	*Turdus merula*	9	1/3[b]	4/6[c]	5/6[d]	0/4
Song Thrush	*Turdus philomelos*	1	1/1[e]			
Robin	*Erithacus rubecula*	13	0/8	0/5		0/3
Dunnock	*Prunella modularis*	2	0/1	0/1		
Blackcap	*Sylvia atricapilla*	2		0/2		0/2
Total		27	2/13	4/14	5/6	0/9

[a] Number of positive DNA detection / total number of bird cultures tested.
[b] Positive PCR from birds No. 2.
[c] Positive PCR from birds Nos. 3, 4, 5 & 6.
[d] Positive PCR from birds Nos. 3 (n=2), 4' (n=1) and 5 (n=2).
[e] Positive PCR from bird No. 8.

All initial tubes containing avian tissues which had been negative by cultivation (n = 35), were screened by PCR. No borrelial DNA was detected in haemocultures (Table 1). However, four culture tubes containing skin samples from 3 additional birds and from one bird captured twice (No. 4) were found positive for *B. garinii* DNA (Table 2). A mixed infection with *B. garinii* and *Borrelia* VS116 was observed in one case (Bird No. 4) (Table 2 and Fig. 1). *Borrelia* infection was present in the skin of juvenile as well as of adult birds.

In summary, borrelial DNA or live spirochetes were observed in BSKII medium containing skin samples from 6/8 (75%) *Turdus* sp. passerines.

Isolation and PCR detection of B. burgdorferi sl in cultures of xenodiagnostic ticks

Five xenodiagnoses were performed on 3 different *T. merula* (Table 3). A total of 11 isolates was obtained from cultivation of 109 xenodiagnostic ticks. Positive results were obtained from 2 blackbirds both captured twice at 15-day intervals and tested twice by xenodiagnosis (Birds Nos. 1, 1' and 4, 4'). Blackbird No. 1 (1') yielded 5 VS116 isolates from 5 xenodiagnostic ticks. Two isolates (NE229 and NE230) showed a typical protein profile for VS116 (OspA of 33.5 kDa), whereas two other VS116 isolates (NE231 and NE249) expressed an OspA of 32 kDa (Fig. 2). Thus, variability was observed in the electrophoretic mobility of OspA in some VS116 isolates from xenodiagnostic ticks. By Western blots, these four VS116 isolates did not react with the

Table 2. Isolation of *Borrelia*, detection and characterization of *Borrelia* DNA in BSK medium inoculated with skin samples of *Turdus* sp.

Bird No.[a]	Skin origin[b]	Isolation	Isolate No.	PCR detection	Species[c]
T. merula					
1	Aspirate / thigh	–		–	
1'	Aspirate / thigh	–		–	
	Aspirate / chin	–		–	
2	Aspirate / thigh	–		+	B. garinii
3	Aspirate / chin	+	NE216	+	B. garinii
	Biopsy / chin L	+	NE219	+	B.garinii & VS116
	Biopsy / chin R	+	NE217	+	B. garinii
4	Aspirate / chin	–		+	B. garinii & VS116
4'	Aspirate / chin	–		–	
	Biopsy / chin L	+	NE225	+	VS116
5	Aspirate / chin	+	NE223	+	VS116
	Biopsy / chin L	+	NE224	+	VS116
	Biopsy / chin R	+	NE218	+	VS116
6	Aspirate / chin	–		+	B. garinii
7	Biopsy / chin L	–		–	
T. philomelos					
8	Aspirate / thigh	–		+	B. garinii

[a] ': recaptured.
[b] L: left-hand side; R: right-hand side.
[c] Species identified by RFLP.

three specific MAbs used. Blackbird No. 4 (4') yielded 5 VS116 isolates and one *B. garinii* isolate from xenodiagnostic ticks. None of these isolates were tested by SDS electrophoresis and Western blotting.

Using Southern blot, we tested three VS116 isolates (NE229, NE230, NE231) that showed fragments at 1.5, 0.8 and 1.9 kbp with the *fla, DnaK* and lpLA7 gene probes, respectively.

All BSK medium tubes containing xenodiagnostic ticks, which had been negative by cultivation, were screened by PCR; *Borrelia* DNA was detected in 32 out of 98 cultures of nymphs (Table 3). DNA of *Borrelia* VS116 (n = 23), *B. afzelii* (n = 3) and of *B. garinii* (n = 2) was detected. Mixed infections were observed in 4 cases, involving always *Borrelia* VS116, and either *B. garinii* (n = 2) or *B. afzelii* (n = 2) (Table 3).

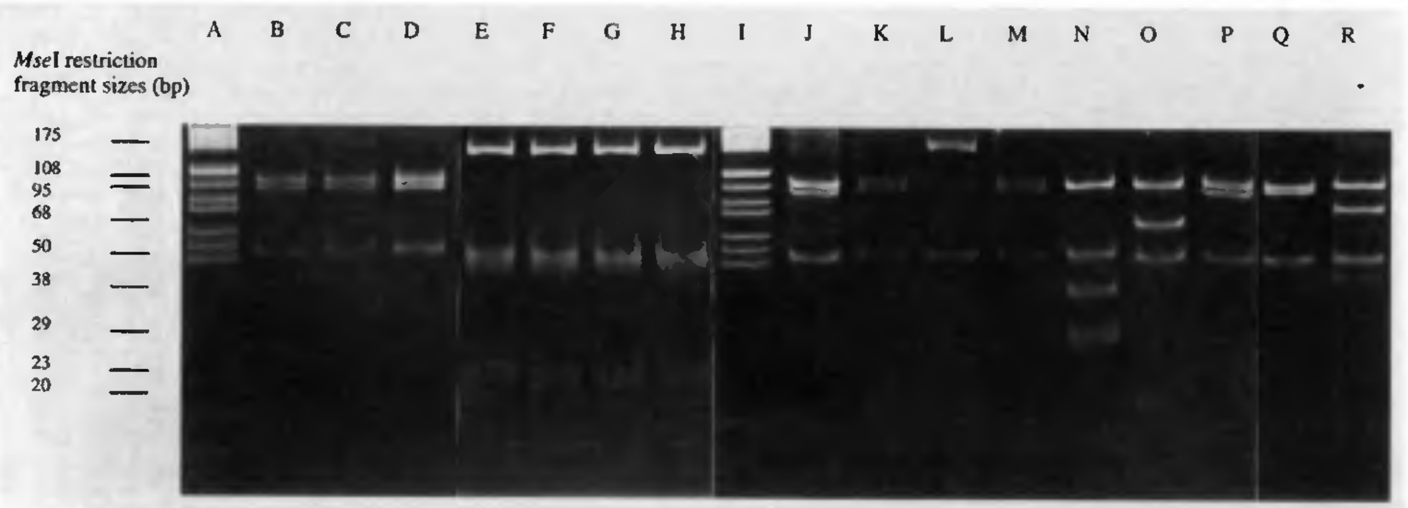

Fig. 1. *Mse*I RFLP patterns of *Borrelia* isolates from *Turdus* sp. Lanes A and I, DNA molecular weight marker V (Boehringer); lanes B to D, isolates NE216, NE219, NE217 from skin aspirate, and skin biopsies of blackbird No. 3, respectively; lanes E to H, isolates NE223, NE224, NE218 from skin aspirate, and skin hiopsies of blackhird No. 5, respectively; lane H, isolate NE225 from skin biopsy of blackhird No. 4'; lane J, *Borrelia* DNA from culture of skin aspirate of blackbird No. 2; lane K, *Borrelia* DNA from culture of skin aspirate of song thrush No. 8; lane L, *Borrelia* DNA from culture of skin aspirate of blackbird No. 4; lane M, *Borrelia* DNA from culture of skin aspirate of blackbird No. 6; lanes N to P, positive controls of *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, respectively; lane Q & R, atypical isolates NE227 & NE228 from field-derived bird-feeding ticks. Light mixed infection occurs in lane C (VS116), and lane L *(B. garinii)*.

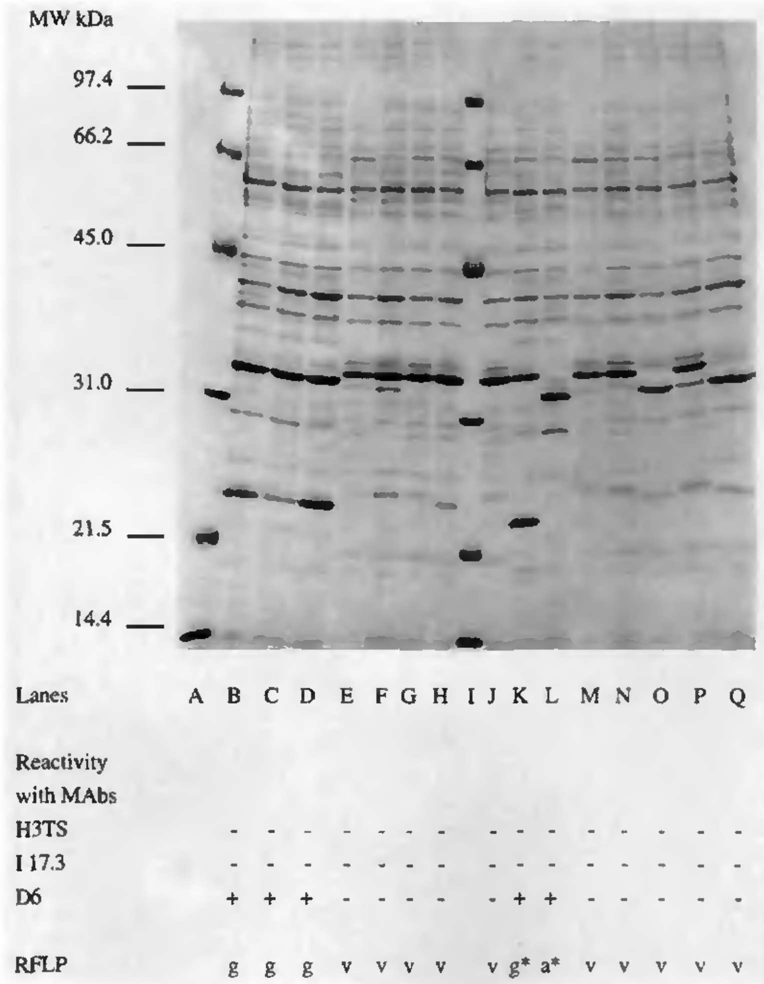

Fig. 2. Protein profiles, Western blotting, and RFLP identification of *Borrelia* isolates from birds, from field-derived bird-feeding ticks and from xenodiagnostic ticks. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi* sensu stricto), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). Lanes A and I, molecular weight standard; lanes B to D, isolates NE219, NE217, NE216 from skin biopsies and skin aspirate of blackbird No. 3, respectively; lanes E to G, isolates NE224, NE218, NE223 from biopsies and skin aspirate of blackbird No. 5, respectively; lane H, isolate NE225 from skin biopsy of blackbird No. 4'; lanes J to M, isolates NE226, NE227, NE228, NE248 from field-derived *I. ricinus* ticks engorged on blackbird No. 1; lane N, isolate NE229 from xenodiagnostic *I. ricinus* tick engorged on blackbird No. 1; lanes O to Q, isolates NE249, NE230, NE231 from xenodiagnostic *I. ricinus* ticks engorged on blackbird No. 1'. +, positive reaction; –, negative reaction. g, *B. garinii*; v, *Borrelia* VS116; a*, atypical RFLP pattern of *B. afzelii* and mixed infection with unknown *Borrelia*; g*, atypical RFLP pattern of *B. garinii*.

Table 3. Isolation of *Borrelia*, and detection and characterization of *Borrelia* DNA in BSK medium inoculated with xenodiagnostic ticks fed on *T. merula*

Bird No.[a]	Isolation				PCR detection in negative cultures							Infection rate (%)[h]	
			RFLP identification[b]				RFLP identification[b]						
	Isolation rate (%)		VS 116	Bg	Detection rate (%)		VS 116	Ba	Bg	VS 116 & Ba	VS 116 & Bg		
1	1/9	(11.1)	1[c]		1/8	(12.5)	1					2/9	(22.2)
1'	4/26	(15.4)	4[d]		9/22	(40.9)	6	2		1		13/26	(50.0)
4	4/35	(11.4)	4[e]		13/31	(41.9)	12			1		17/35	(48.6)
4'	2/20	(10.0)	1[f]	1[g]	8/18	(44.4)	4	1	1		2	10/20	(50.0)
7	0/19	(0.0)			1/19	(5.3)			1			1/19	(5.3)
Total	11/109	(10.1)	10	1	32/98	(32.7)	23	3	2	2	2	43/109	(39.4)

[a] ': recaptured.
[b] Ba: *Borrelia afzelii*; Bg: *Borrelia garinii*.
[c] Isolate No. NE229.
[d] Isolates No. NE230, NE231, NE249, NE253.
[e] Isolates No. NE254, NE255, NE256, NE257.
[f] Isolate No. NE258.
[g] Isolate No. NE259.
[h] determined by both methods.

In summary, all birds on which xenodiagnosis was performed transmitted spirochetes to 5 to 50% of feeding ticks according to both isolation and PCR results (Table 3).

Isolation and PCR detection of B. burgdorferi sl in cultures of field-derived bird-feeding ticks

The cultivation of 7 molted field-derived *I. ricinus* ticks, engorged as larvae (n = 3) and nymphs (n = 4) on blackbird No. 1, yielded 2 isolates from larvae (NE226 and NE227), and 2 from nymphs (NE228 and NE248). Two isolates (NE226 and NE248) were identified as *Borrelia* VS116 by RFLP analysis, and presented typical protein profiles with an OspA of 33.5 kDa and no reaction to the three species-specific MAbs (Fig. 2). The other 2 isolates (NE227 and NE228) each showed atypical RFLP patterns (Fig. 1, lanes Q and R). NE227 was identified as an atypical *B. garinii*, with a 95bp DNA fragment slightly larger than usual. NE228 was determined as an atypical *B. afzelii*, with a 68bp fragment larger than usual, mixed with *Borrelia* showing an unidentified profile. Both isolates reacted with MAb D6 specific of *B. garinii*, but NE227 expressed an OspA of 33.5 kDa like VS116 *Borrelia*, and NE228 presented an OspA of 32 kDa like *B. afzelii* but without OspB (Fig. 2). Thus, variability was observed in the electrophoretic mobility of OspA in one *B. garinii* isolate from a field-derived bird-feeding tick.

Using Southern blot, isolate NE226 exhibited typical DNA fragments of the VS116 genogroup, and isolate NE227 reacted as a typical *B. garinii* with the *fla, DnaK* and lpLA7 gene probes. Other isolates were not tested by Southern blotting.

All negative initial cultures of field-derived ticks collected from bird No. 1 (n = 3) were screened by PCR and remained negative for detection of borrelial DNA.

Isolation of B. burgdorferi sl in cultures of free-living ticks

The cultivation of 94 host-seeking *I. ricinus* (54 nymphs, 18 females, and 22 males) yielded 24 borrelial isolates: 12 from nymphs, 7 from females and 5 from males. Infection in free-living ticks was heterogeneous. *B. burgdorferi* ss was isolated from one female and one male, *B. garinii* from 4 females and 3 males. All 12 nymphs as well as one female and one male carried *B. afzelii*, and group VS116 was isolated from one female. Fig. 3 illustrates protein profiles and reactions to MAbs of isolates from free-living ticks. Phenotyping results using SDS-PAGE and Western blot corroborated genotyping findings. However, we observed an electrophoretic mobility of OspA at 32 kDa for 4 *B. garinii* isolates (NE210, NE214, NE215, NE233), and at 31 kDa for one VS116 isolate (NE246). Free-living tick isolates were not tested by Southern blotting.

In summary, *B. garinii* was isolated from the skin of one bird and VS116 spirochetes from the skin of 2 birds. RFLP analysis of negative cultures of skin

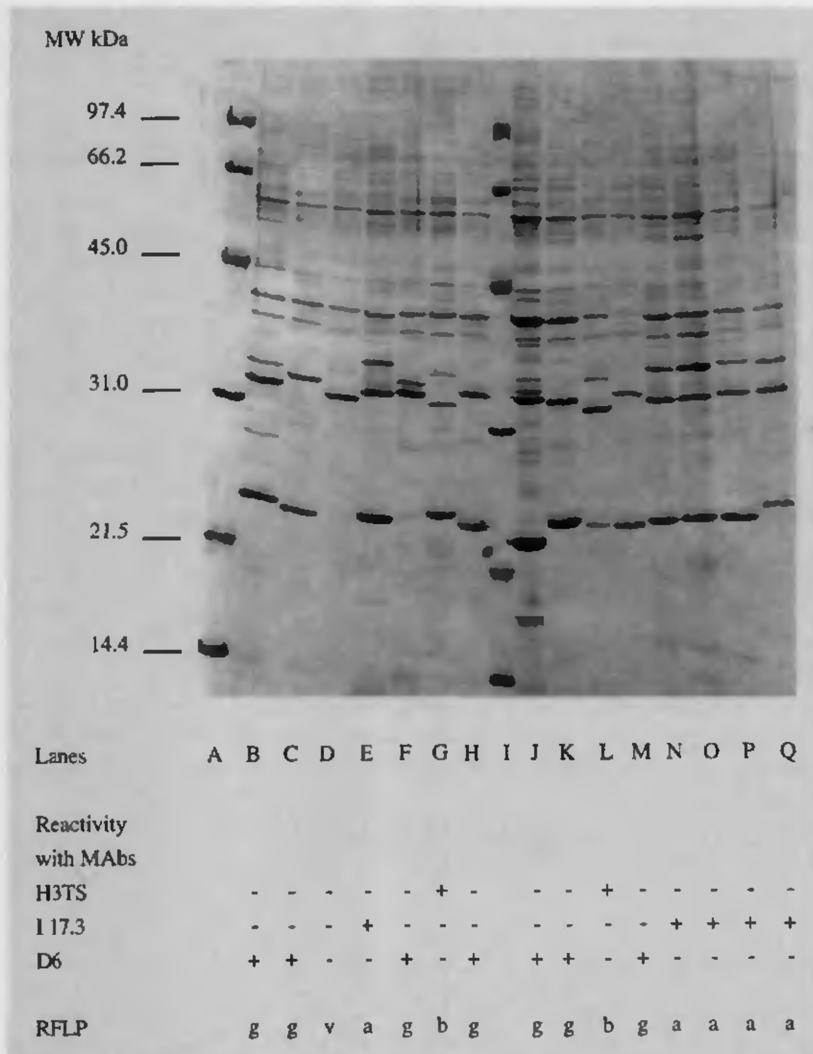

Fig. 3. Protein profiles, Western blotting, and RFLP identification of *Borrelia* isolates from free-living ticks. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi* sensu stricto), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). Lanes A and I, molecular weight standard; lanes B to H, isolates NE210, NE211, NE246, NE212, NE233, NE234, NE235 from *I. ricinus* females, respectively; lanes J to M, isolates NE214, NE215, NE247, NE236 from *I. ricinus* males, respectively; lanes N to Q, isolates NE171, NE191, NE192, NE194 from *I. ricinus* nymphs, respectively. +, positive reaction; –, negative reaction.
a, *B. afzelii*; b, *B. burgdorferi* sensu stricto; g, *B. garinii*; v, *Borrelia* VS116.

samples confirmed the presence of VS116 but revealed a mixed infection with *B. garinii* in the skin of one of these birds and *B. garinii* was detected in the skin of 3 additional birds. Thus, using both methods, *Borrelia* infection was detected in 6/8 birds. Results of xenodiagnosis on 3 birds showed that VS116 was mainly transmitted to ticks by 2 birds, and that one of them also transmitted *B. garinii* to ticks. RFLP analysis of negative cultures of xenodiagnostic ticks confirmed that VS116 was frequently transmitted to ticks. The transmission of *B. garinii* to xenodiagnostic ticks was also confirmed. However, *B. afzelii* which had not been isolated or detected by PCR from skin samples or isolated from xenodiagnostic ticks, was revealed by PCR/RFLP in negative cultures of xenodiagnostic ticks fed on 2 different birds.

Discussion

In the present study, we focused on a target group of passerines, blackbirds and thrushes being frequently infested with *I. ricinus* ticks, to investigate their role in the transmission cycle of *B. burgdorferi* sl using xenodiagnosis. Two complementary methods were used to detect the infection: isolation in BSK medium and detection of borrelial DNA by PCR in BSK medium containing avian skin or ticks. The use of PCR to screen BSK medium improved the detection rate and therefore provided a better estimate of the infection prevalence in hosts and in ticks.

Our results demonstrated that *Turdus* sp. can be infected by spirochetes of the Lyme borreliosis complex. Borrelial DNA or live spirochetes were detected in BSK cultures of skin samples from 6/8 (75%) *Turdus* sp.. This value is not significantly different from the proportion of *Turdus* sp. parasitized by *Borrelia*-infected ticks (10/19, 53%) found in a previous study in the same area (17). Our observation corroborates previous findings that *B. burgdorferi* sl can be isolated from avian tissues (3, 17, 24).

The results of xenodiagnosis have clearly demonstrated that *Turdus* sp. can transmit *B. burgdorferi* sl to uninfected feeding ticks. The prevalence of infection in xenodiagnostic ticks varied from 5 to 50%, with a mean value of 39% (43/109). This percentage is not significantly different from the proportion of infected field-derived ticks (29/105, 28%) collected on *T. merula* in the same endemic area (17).

Recapture of birds at 15-day intervals showed that reisolation from birds was possible and that they remained infective for ticks during this period. Infection was present in juvenile as well as in adult passerines suggesting a long-term persistence of *B. burgdorferi* sl in avian hosts. These observations provide evidence that certain bird species act as reservoirs for *B. burgdorferi* sl in an endemic focus. This had been previously suspected by various field workers in Europe, North America and Japan, but never clearly shown (3, 4, 14, 17, 20, 26–28, 34).

The genotypic and phenotypic analyses performed on *Borrelia* isolates from birds demonstrated that skin of *Turdus* sp. from this endemic area was infect-

ed by *B. garinii* or *Borrelia* VS116, and that a mixed infection with these 2 genospecies can occur in the same skin sample. Previously, spirochetes of group VS116 were only isolated or detected from ticks (29, 32) and not from vertebrate hosts. The pathogenicity of VS116 to humans remains unknown at present.

The results of cultivating xenodiagnostic ticks demonstrated that blackbirds transmitted *Borrelia* VS116 and *B. garinii* to feeding ticks. This allowed us to identify one of the reservoir hosts of VS116. Using PCR, we showed that blackbirds also transmitted *B. afzelii* to feeding ticks. Interestingly, *B. afzelii* was not present in any of the bird skin samples, but it was present in xenodiagnostic ticks. The cultivation of this species was unsuccessful and only *B. afzelii* DNA was detected in culture. Hence, the transmission of live *B. afzelii* from birds to ticks remains uncertain. In xenodiagnostic ticks, a low level (4/43, 9%) of mixed infections occurred, always involving VS116. Anyway, *Borrelia* VS116 was the genotypic group most frequently found (37/43, 86%); *B. garinii* (5/43, 12%) and *B. afzelii* (5/43, 12%) seem to be transmitted less efficiently from birds to ticks. In view of our observations, the results of a previous study in Europe (23) showing the incompetence of blackbirds, were probably due to the *Borrelia* genospecies used in the experiment. In our study, *B. burgdorferi* ss was found neither in the skin of birds nor in xenodiagnostic ticks, although this species was present in the free-living tick population. This could explain why catbirds were found incompetent reservoirs in North America (21), where *B. burgdorferi* ss is present but where VS116 and *B. garinii* are absent.

The protein profile analysis of *Borrelia* showed that isolates obtained from birds were typical *B. garinii* or *Borrelia* VS116, with constant Osp molecular weights. By contrast, the *B. garinii* and *Borrelia* VS116 isolates obtained from ticks (bird-feeding and free-living ticks) showed an electrophoretic variability of the OspA. This suggests that birds may tend to "clone" strains, whereas ticks seem to induce protein changes (10, 13) and generate subtypes that are frequent in *B. garinii* (39). Interestingly, the same phenomenon was observed in isolates obtained from rodents (15) and isolates obtained from ticks fed on rodents (11).

PCR allowed us to detect DNA of spirochetes in BSK medium containing tick or skin samples from which no borreliae could be recovered by isolation. However, DNA of living as well as dead organisms can be detected. This last point must be kept in mind when analyzing data obtained with PCR, because the understanding of phenomena occurring in the ecology of Lyme borreliosis might be misinterpreted. However, isolation alone would have underestimated the presence of *Borrelia* in ticks and avian skin. The use of PCR in the present work not only increased the sensitivity of detection of *B. burgdorferi* sl in this material, but also confirmed the results obtained by isolation. Thus, both methods used together allowed a better evaluation of the transmission of spirochetes between birds and ticks.

An intimate association seems to exist between birds and *Borrelia* VS116 and to a lesser extent, *B. garinii*, similar to the association occurring between small rodents and *B. afzelii* (11, 15). This observation emphasizes the hypothesis that

specific enzootic transmission cycles maintain the different *B. burgdorferi* sl genospecies in nature, as suggested by *Nakao* et al. (26) and *Humair* et al. (15). Hosts may get infected by various genospecies, but for unknown reasons, hosts acting as filters seem to be predisposed to mainly transmit one genospecies to ticks, to the detriment of other genomic groups. The presence of *B. garinii* and VS116 in birds might be related to a better affinity of these genospecies to the high body temperature of these hosts (38). In North America, the prominent genospecies, *B. burgdorferi* ss is mainly maintained by *Peromyscus leucopus* reservoirs. The two other genomic groups recently described in the United States (31), involving other tick vectors and vertebrate hosts, show their own enzootic cycles. In Europe, where different genospecies are almost equally present in enzootic areas, multiple transmission cycles occur simultaneously. In enzootic foci in Switzerland, small rodents are main reservoirs of *B. afzelii* (11, 15), and birds are main reservoirs of *Borrelia* VS116 and *B. garinii*. Accordingly, as *I. ricinus* larvae feed more readily on small rodents, *B. afzelii* infection subsequently predominates in the free-living nymph population, as observed here at our study site and other enzootic areas in Switzerland (*Gern*, unpublished data). Similarly, *I. ricinus* nymphs frequently infest avian hosts, and *B. garinii*, being associated with birds, is more frequently isolated from the resulting host-seeking adults than from host-seeking nymphs (13).

Further investigations on the relationship between Lyme borreliosis genospecies and reservoir hosts, especially between birds and *B. burgdorferi* ss in the USA, where VS116 and *B. garinii* are absent, are needed to clearly understand the role of birds in the ecology of Lyme borreliosis. In Europe, the maintenance of *B. burgdorferi* ss in nature should be particularly explored.

Acknowledgements. This research was supported by the Swiss National Science Foundation (No. 32-29964-90), the Swiss Federal Office of Education and Science (No. 93-0363), and the European Union Biomedical and Health Research (Biomed I) Programme (No. BMH-CT93-1183).

We thank *Christiane Brenner, Raquel Martinez* and *Olivier Rais* for their excellent technical assistance, *Guy Baranton, Alan Barbour* and *Olivier Péter* for the monoclonal antibodies, and *Patricia Nuttall* for her comments on a previous version of the manuscript. This work is part of the PhD dissertation of P.F.H.

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Note:
Since this manuscript was submitted, *Borrelia* VS116 has been named *Borrelia valaisiana* (Wang, G., A. P. van Dam, A. Le Fleche, D. Postic, O. Péter, G.Baranton, R. de Boer, L. Spanjaard, and J. Dankert. Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). Int. J. Syst. Bacteriol. 47 (1997) 926–932) and *Borrelia* PotiB2 has been named *Borrelia lusitaniae* (Le Fleche, A., D. Postic, K. Girardet, O. Péter, and G. Baranton. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int. J. Syst. Bacteriol. 47 (1997) 921–925).

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Acta Tropica 69 (1998) 213–227

ACTA TROPICA

Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland

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Received 6 September 1997; accepted 17 October 1997

Abstract

The infection and reservoir status of red squirrels (*Sciurus vulgaris*) for *Borrelia burgdorferi* sensu lato were studied in Switzerland. *B. burgdorferi* sensu lato was isolated from 15 skin samples from 4/6 dead red squirrels, victims of road traffic. Isolates were identified using restriction fragment length polymorphism (RFLP): *B. burgdorferi* sensu stricto was present in 14 culture tubes containing skin samples and *B. afzelii* in two other tubes. A mixed infection was revealed in one case. A total of 227 ticks attached to squirrels were cultivated in BSKII medium and 90 isolates were obtained. Genotypic identification by RFLP showed that *B. afzelii* (59%) and *B. burgdorferi* sensu stricto (46%) dominated in ticks feeding on red squirrels. Data collected from one particular animal, highly infested with *Ixodes ricinus* and harbouring numerous *Borrelia*-infected *Ixodes ricinus* ticks, showed that transmission of *B. burgdorferi* sensu lato occurred from *S. vulgaris* to feeding ticks. More precisely, *B. burgdorferi* sensu stricto and *B. afzelii* were mainly transmitted from *S. vulgaris* to ticks. The present data emphasized the results obtained previously from small rodents and birds in Japan and in Switzerland, showing the occurrence of specific associations between host species and *Borrelia* genospecies. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Lyme borreliosis; Ecology; *Sciurus vulgaris*; Host; *Ixodes ricinus*; *Borrelia burgdorferi* sensu lato

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PII S0001-706X(97)00126-5

1. Introduction

Lyme borreliosis is a tick-borne disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato (sl) complex. In Europe, this complex includes three genospecies pathogenic for humans, *B. burgdorferi* sensu stricto (ss), *B. afzelii* and *B. garinii* (Johnson et al., 1984; Baranton et al., 1992; Canica et al., 1993) and two newly described genomic groups apparently non-pathogenic for humans, group VS116 and group PotiB2 (Péter and Bretz, 1992; Postic et al., 1994). These borreliae are commonly vectored by *Ixodes ricinus* in European endemic areas. Lyme borreliosis spirochetes are mainly maintained in nature through a transmission cycle involving ticks and vertebrates that act as reservoirs. Ecological studies on Lyme borreliosis conducted in Europe have shown that some mammal and bird species frequently infested by *I. ricinus* transmit infection to ticks. Mammals, the most extensively studied, are small rodents, especially *Apodemus* mice and *Clethrionomys* voles (Aeschlimann et al., 1986; Matuschka et al., 1992; De Boer et al., 1993; Humair et al., 1993; Tälleklint and Jaenson, 1994; Humair et al., 1995; Kurtenbach et al., 1995). Medium-sized rodents, like edible dormice (*Glis glis*) (Matuschka et al., 1994), rats (*Rattus norvegicus*) (Matuschka et al., 1996) and grey squirrels (*Sciurus carolinensis*) (Craine et al., 1997) have been determined to be reservoirs as well. Insectivores like *Neomys* and *Sorex* shrews (Tälleklint and Jaenson, 1994) and hedgehogs (*Erinaceus europaeus*) (Gray et al., 1994; Gern et al., in prep), as well as lagomorphs (*Lepus europaeus* and *L. timidus*) (Tälleklint and Jaenson, 1994) are also implicated. Besides mammals, some bird species like pheasants (*Phasianus colchicus*) and *Turdus* sp. passerines and migrating birds may act as reservoirs for *B. burgdorferi* sl (Olsén et al., 1995; Craine et al., 1997; Humair et al., in press; Kurtenbach et al., in press).

In this study, we tried to evaluate the infection and reservoir status of the European indigenous red squirrel, *Sciurus vulgaris*, in endemic areas in Switzerland. For this purpose, *B. burgdorferi* sl was isolated from these hosts and from squirrel-feeding ticks. Isolates were identified using restriction fragment length polymorphism (RFLP) analysis to demonstrate if red squirrels were infected by a particular genospecies.

2. Materials and methods

2.1. Collection of squirrels and feeding ticks

Dead red squirrels (*S. vulgaris*), victims of road traffic, were collected at different locations near Neuchâtel, Switzerland (Canton of Neuchâtel and Canton of Jura, at various altitudes, Table 1), and were brought to the laboratory. They were examined for ticks and skin samples were taken for spirochete isolation. Ticks were removed by forceps, counted, identified to species, stage and sex and were maintained in labelled vials at room temperature and 95% humidity until use for cultivation. Some ticks, which were fully engorged, were held in the conditions

mentioned above until moult was completed and were inoculated into BSKII medium for isolation of spirochetes.

2.2. Collection of free-living ticks

Host-seeking *I. ricinus* nymphs and adults were collected by flagging the vegetation at the site of collection of squirrel No. 1 (Neuchâtel) during June 1995. Ticks were identified to species, stage and sex and were maintained as mentioned (Section 2.1) until use for spirochete isolation. Flagging sessions were not planned at each squirrel collection site, since the three most important genospecies were shown to be present in enzootic areas in Switzerland (Hu et al., 1994; Humair et al., 1995; Péter et al., 1995; Gern et al., in prep).

2.3. Isolation of spirochetes

Skin necropsies were collected from each squirrel to isolate spirochetes. Skin samples were systematically removed from left and right ears and occasionally from chin, throat and axillae. Skin samples were taken with sterilized small sharp scissors after shaving and cleaning the skin with 70% ethanol. Needle aspiration of skin described by Piesman et al. (1991) was also used in one case (squirrel No. 2). Blood, urine, synovial fluid and tissue samples from liver, heart, spleen, urinary bladder and kidney were also removed in another case (squirrel No. 1). Skin necropsies, aspirate and tissue samples were immediately inoculated into tubes containing BSKII medium supplemented according to Sinsky and Piesman (1989). Partially engorged, fully engorged and moulted ticks collected from squirrels and free-living

Table 1
Ixodes ricinus ticks on red squirrels (*Sciurus vulgaris*) collected near Neuchâtel, Switzerland

Animal number	Sex	Age	Collection			No. *I. ricinus* ticks		
			Locality	Altitude (m)	Date	Larvae	Nymphs	Females
1	M	Ad	Neuchâtel[a]	620	March 1994	1	46	0
2	M	Ad	Neuchâtel[b]	540	April 1996	6	87	0
3	M		Cerneux-Veusil	1020	April 1996	0	0	0
4	F		Rochefort	840	June 1996	31	50	0
5	M	Juv	Neuchâtel[b]	640	June 1996	369	377	1
6	M	Ad	Hauts-Geneveys	1045	June 1996	2	10	1
Total						409	570	2

F, female; M, male; Ad, adult; Juv, juvenile.
[a] Bois de l'Hopital.
[b] Voens.

ticks were briefly soaked in 70% ethanol and squashed with sterilized forceps in tubes containing BSKII medium modified by Sinsky and Piesman (1989).

Inoculated cultures were screened by dark-field microscopy for the presence of spirochetes after 10 days and 3, 4, 6 and 8 weeks of incubation at 34°C. Spirochetes in culture tubes were analyzed by SDS-PAGE, Western blot and RFLP.

2.4. SDS-PAGE and Western blot

After 2–5 subcultures, squirrel and tick isolates were analyzed by SDS-PAGE and Western blotting as described elsewhere (Humair et al., in press). For Western blots, monoclonal antibodies (MAbs) were used for specific identification of borreliae: MAb H3TS recognizes the OspA (31 kDa) of *B. burgdorferi* ss (Barbour et al., 1985), MAb I 17.3 reacts with the OspB (35 kDa) of *B. afzelii* (Canica et al., 1993), and MAb D6 identifies a 12 kDa protein of *B. garinii* (Péter and Bretz, 1992).

2.5. RFLP analysis

The variable intergenic spacer between repeated 23S (*rrl*)—5S (*rrf*) ribosomal genes of *B. burgdorferi* sl was used as a template for polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) analysis with *Mse*I endonuclease, as described by Postic et al. (1994). Two ml of initial cultures were used to detect *Borrelia* DNA in positive cultures, the whole culture volume (4 ml) was used to detect *Borrelia* DNA in culture tubes negative after 8 week incubation at 34°C.

3. Results

3.1. Tick infestation of squirrels

Immature and adult stages of *I. ricinus* were removed from five out of six squirrels (Table 1). Immatures were far more numerous than adults on these medium-sized rodents, since a total of 409 larvae (41.7%), 570 nymphs (58.1%) and only two females (0.2%) were collected. One squirrel (No. 5), harbouring a total of 747 *I. ricinus* ticks (369 larvae, 377 nymphs and 1 female) showed that *S. vulgaris* could be highly infested. Two squirrels collected at higher altitude (>1000 m) showed fewer ticks (No. 6) or no tick at all (No. 3). Squirrels may get infested by ticks early in the year (mid-March) as observed for squirrel No. 1. *I. ricinus* ticks were found attached on the ears, chin and throat and also in the axillae in the case of the highly infested squirrel (No. 5). On this animal, feeding ticks were distributed in clusters and red skin lesions were observed around clusters, suggesting that an immunologic response to tick infestation and/or to spirochete infection occurred.

Table 2
Isolation and DNA detection of *Borrelia* genospecies in BSK medium inoculated with skin samples of *Sciurus vulgaris*

Animal number	Skin origin[a]	Isolate number	PCR	Species[b]
1	Necropsy/ear L	NE 63	+	Bb
	Necropsy/ear R	NE 59	+	Bb
2	Necropsy/ear L	NE 260	+	Bb[c]
	Necropsy/ear R	NE 261	+	Bb
	Necropsy/throat	NE 262	+	Ba
	Aspirate/ear	RE 263	+	Bb[c]
	Aspirate/throat		–	
3	Necropsy/ear L		+	Bg
	Necropsy/ear R		–	
4	Necropsy/ear L_1		–	
	Necropsy/ear L_2		–	
	Necropsy/ear R_1		–	
	Necropsy/ear R_2		–	
	Necropsy/throat		–	
5	Necropsy/ear L	NE 264	+	Bb
	Necropsy/ear R_1	NE 265	+	Bb
	Necropsy/ear R_2		+	Ba
	Necropsy/chin	NE 266	+	Bb and Ba
	Necropsy/throat	NE 267	+	Bb
	Necropsy/axilla	NE 268	+	Bb
6	Necropsy/ear L_1	NE 269	+	Bb
	Necropsy/ear R	NE 270	+	Bb
	Necropsy/chin	NE 271	+	Bb[c]
	Necropsy/ear L_2	NE 272	+	Bb

[a]L, left-hand side; R, right-hand side.
[b]Species identified by RFLP: Ba, *Borrelia afzelii*; Bb, *B. burgdorferi* sensu stricto.
[c]Atypical RFLP pattern.

3.2. Isolation and DNA detection of B. burgdorferi sl from squirrel skin samples

A total of 15 isolates were obtained from cultivation of 24 squirrel skin samples taken from six animals (Table 2). Spirochetes were isolated from ears, chin, throat and axillae. Isolates were named according to our laboratory denomination (NE). These isolates were identified by RFLP as *B. burgdorferi* ss ($n = 13$), *B. afzelii* ($n = 1$) and one isolate presented a mixed infection with *B. burgdorferi* ss and *B. afzelii* (Table 2). Three *B. burgdorferi* ss isolates exhibited an atypical DNA pattern with a fragment of 40 bp instead of 38 bp. No spirochetes were detected in culture tubes containing samples of blood, urine, synovial fluid, liver, heart, spleen, urinary bladder and kidney from squirrel No. 1, although ear skin necropsies from this host were positive. An important growth of contaminants—probably preventing successful cultivation of spirochetes—was observed in culture tubes from squirrel No.

4. This could be due to the late delivery of this animal into our laboratory (> 24 h after collection).

Protein profiles and Western blots using species-specific MAbs of squirrel isolates confirmed the identification obtained by RFLP (Fig. 1). Squirrel isolates showed usual protein profiles for *B. burgdorferi* ss (OspA of 31 kDa and OspB of 34 kDa)

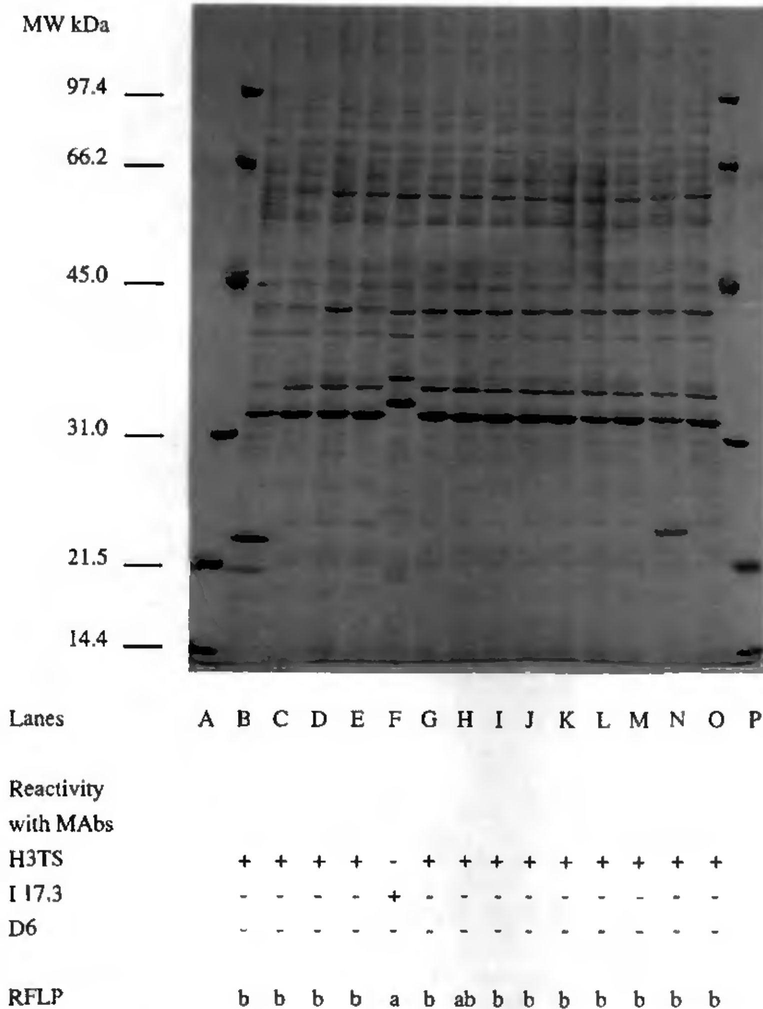

Fig. 1. Protein profiles, Western blotting and RFLP identification of *Borrelia* isolates from *Sciurus vulgaris*. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi* sensu stricto), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). Lanes A and P, molecular weight standard; lane B, isolate NE 63 from squirrel No. 1; lanes C–F, isolates NE 260, NE 261, NE 263 and NE 262 from squirrel No. 2; lanes G–K, isolates NE 264–268 from squirrel No. 5; lanes L–O, isolates NE 269–272 from squirrel No. 6. +, positive reaction; –, negative reaction. a, *B. afzelii*; b, *B. burgdorferi* sensu stricto.

and for *B. afzelii* (OspA of 32 kDa and OspB of 35 kDa). Proteins in the OspC region exhibited strong expression in two cases only (lanes B and N). Mixed infection detected by RFLP in isolate NE266 (No. 5) was not revealed, neither by SDS electrophoresis nor by Western blot, suggesting that subcultures needed for these two latter methods selected one of the genospecies (*B. burgdorferi* ss, lane H).

Cultures of skin samples from animals Nos. 2, 3, 4 and 5, which remained negative after 8 week incubation at 34°C, were screened by PCR and *Borrelia* DNA was amplified in two tubes. RFLP analysis revealed *B. garinii* and *B. afzelii* in culture tubes containing skin samples from squirrels Nos. 3 and 5, respectively (Table 2).

In summary, *B. burgdorferi* sl was isolated or PCR detected in skin samples from 5/6 (83%) *S. vulgaris*, and *B. burgdorferi* ss dominated in squirrel skin cultures followed by *B. afzelii*.

3.3. Isolation of B. burgdorferi sl from ticks fed on squirrels

A total of 227 partially engorged or moulted *I. ricinus* ticks (16 larvae and 211 nymphs), fed on five *S. vulgaris*, were inoculated into BSK medium and 90 isolates (39.6%) were obtained (Table 3). A total of 81 isolates were cultivated from 211 nymphs (38.4%) and nine isolates were obtained from 16 larvae (56.2%). The isolation rate from squirrel-feeding ticks varied from 3.3 to 69.5%. A high isolation rate of spirochetes was obtained from *I. ricinus* larvae (64.3%) and nymphs (69.5%) from squirrel No. 5. The genotypic identification by RFLP of the 90 isolates showed that *B. afzelii* ($n = 43$) and *B. burgdorferi* ss ($n = 31$) were the two genospecies most frequently found in ticks fed on squirrels. *B. garinii* was rare ($n = 4$). Mixed infection concerned only *B. afzelii* and *B. burgdorferi* ss ($n = 10$). Considering mixed infections, *B. afzelii* was found in 58.9% of infected ticks and *B. burgdorferi* ss in 45.6% of infected ticks.

Phenotyping results using SDS-PAGE and Western blot with species-specific MAbs corroborated genotyping findings (Fig. 2). Most *B. afzelii* isolates ($n = 11$) showed a typical pattern with an OspA of 32 kDa and an OspB of 35 kDa. However, we observed an electrophoretic mobility of OspB at 35.5 kDa for three *B. afzelii* isolates (lanes F, J and U). *B. garinii* isolates tested by SDS-PAGE and Western blot showed an atypical electrophoretic mobility of OspA (lanes K and V). Independent of the genospecies, proteins in the OspC region were present or not.

3.4. Isolation of B. burgdorferi sl from free-living ticks

The cultivation of 118 free-living *I. ricinus* ticks (90 nymphs, 17 females, 11 males) from Neuchâtel collection site yielded 29 isolates: 21 from 90 nymphs (23.3%), seven from 17 females (41.2%) and one from 11 males (9.1%). RFLP analysis of these isolates showed that *B. afzelii* was obtained from 11 nymphs and two females, *B. burgdorferi* ss from two nymphs and three females, *B. garinii* from seven nymphs and two females, VS116 from one nymph. A mixed infection with *B. afzelii* and *B. garinii* was observed in the only male isolate.

Table 3
Isolation of *Borrelia* genospecies in BSK medium inoculated with *Ixodes ricinus* ticks fed on *Sciurus vulgaris*

Animal number	Tick stages infesting hosts	Isolation rate (%)	RFLP identification				
			Bb	Ba	Bg	Bb and Ba	Unidentified
1	N	1/30 (3.3)	1				
2	N	20/67 (29.9)	3[c]	16	1		
	L	0/1					
4	N	3/31 (9.7)	1[c]	1	1		
	L	0/1					
5	N	57/82[a] (69.5)	23[c]	22	2	9	1
	L	9/14[b] (64.3)	3	4		1	1
6	N	0/1					
Total		90/227 (39.6)	31	43	4	10	2

L, larvae; N, nymphs; Ba, *Borrelia afzelii*; Bb, *B. burgdorferi* sensu stricto; Bg, *B. garinii*.
[a]3 Isolates were obtained from moulted nymphs.
[b]5 Isolates were obtained from moulted larvae.
[c]Atypical RFLP pattern observed in 3, 1 and 1 isolates from squirrels No. 2, 4 and 5, respectively.

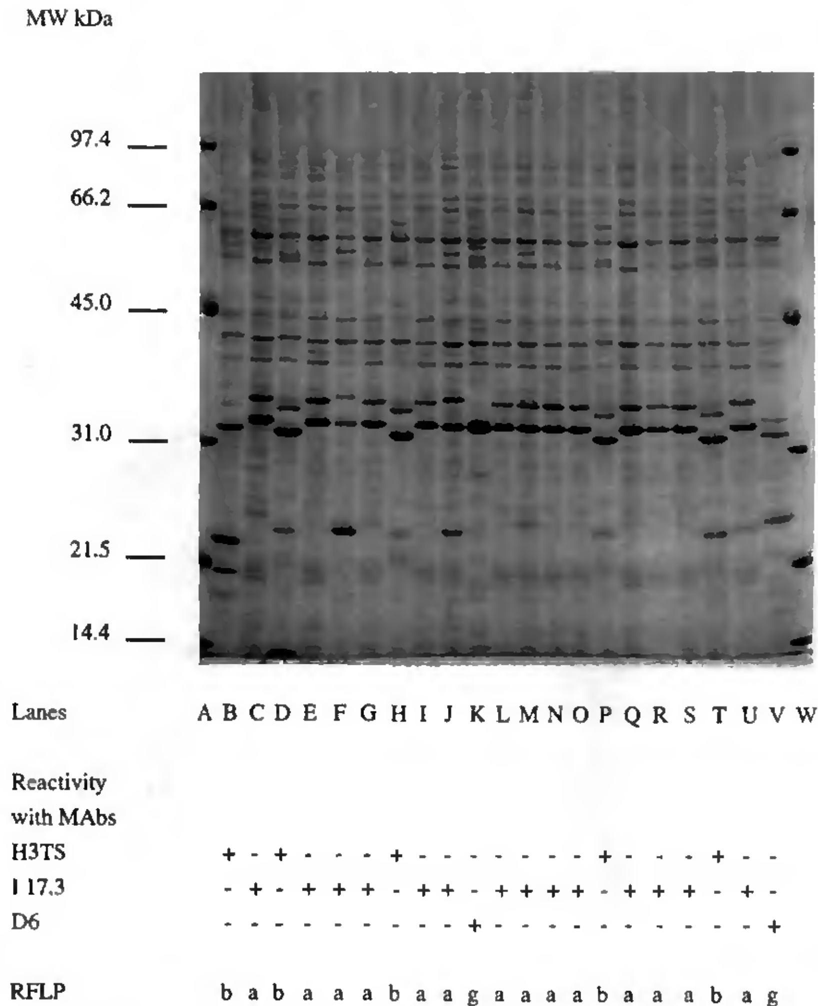

Fig. 2. Protein profiles, Western blotting and RFLP identification of *Borrelia* isolates from field-derived ticks feeding on *Sciurus vulgaris*. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi* sensu stricto), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). Lanes A and W, molecular weight standard; lane B, isolate NE 64 from *Ixodes ricinus* fed on squirrel No. 1; lanes C–S, isolates NE 273–275 and NE 278–291 from *I. ricinus* fed on squirrel No. 2; lanes T–V, isolates NE 292–294 from *I. ricinus* fed on squirrel No. 4. +, positive reaction; −, negative reaction. a, *B. afzelii*; b, *B. burgdorferi* sensu stricto; g, *B. garinii*.

Species-specific MAbs confirmed the identification by RFLP (Fig. 3A,B). Protein profiles showed atypical electrophoretic mobility of Osps for some isolates: OspB at 35.5 kDa for eight *B. afzelii* isolates (Fig. 3A: lanes B, E, H, M, N, P and Q; Fig. 3B: lane D) and OspA for eight *B. garinii* varying between 31 and 33 kDa (Fig. 3A: lanes D, G, K, L, O, T; Fig. 3B: lanes F and H). Proteins in the OspC area were always present.

4. Discussion

B. burgdorferi sl is maintained in nature through a transmission cycle involving ticks and hosts. Although transovarial transmission exists (Zhioua et al., 1994; Bellet-Edimo, 1997), Lyme borreliosis spirochetes need hosts and transstadial

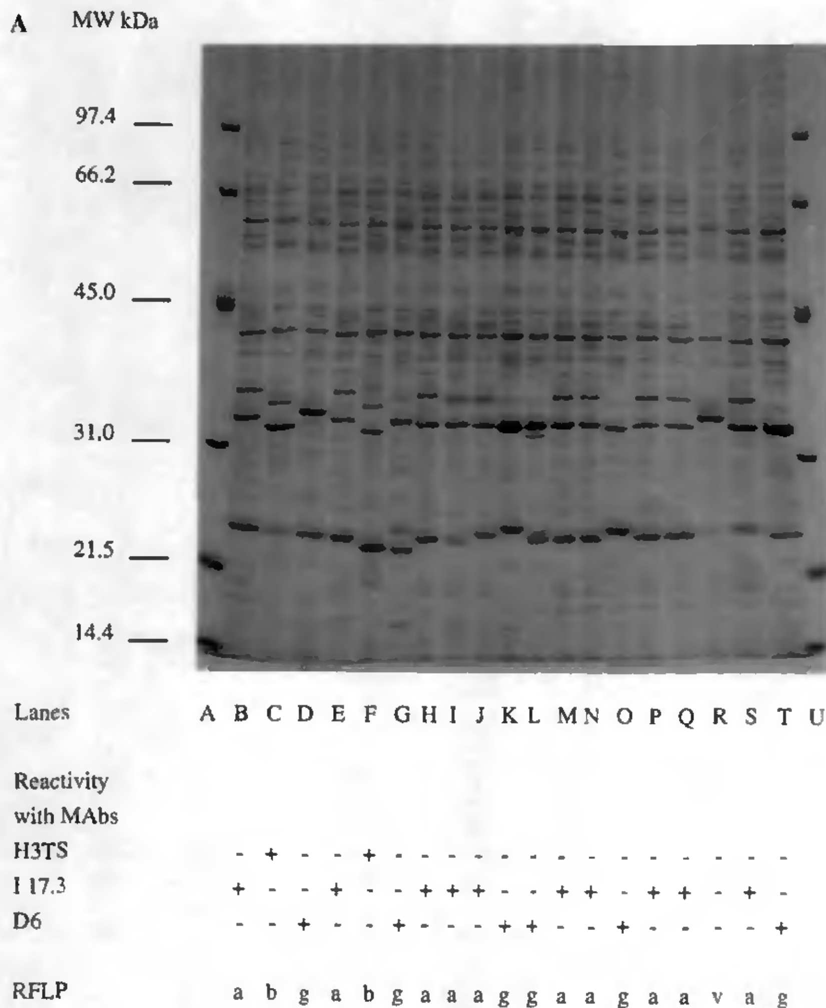

Fig. 3. (A and B) Protein profiles, Western blotting and RFLP identification of *Borrelia* isolates from free-living *Ixodes ricinus* ticks. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi* sensu stricto), I 17.3 (anti-*B. afzelii*) and D6 (anti-*B. garinii*). (A): Lanes A and U, molecular weight standard; lane B–T, isolates NE 125–130 and NE 158–170 from *I. ricinus* nymphs. (B): Lane A, molecular weight standard; lane B to G, isolates NE 172, NE 205–206, NE 232, NE 208–209 from *I. ricinus* females; lane H, isolate NE 213 from *I. ricinus* male. +, positive reaction; −, negative reaction. a, *B. afzelii*; b, *B. burgdorferi* sensu stricto; g, *B. garinii*; v, *Borrelia* VS116.

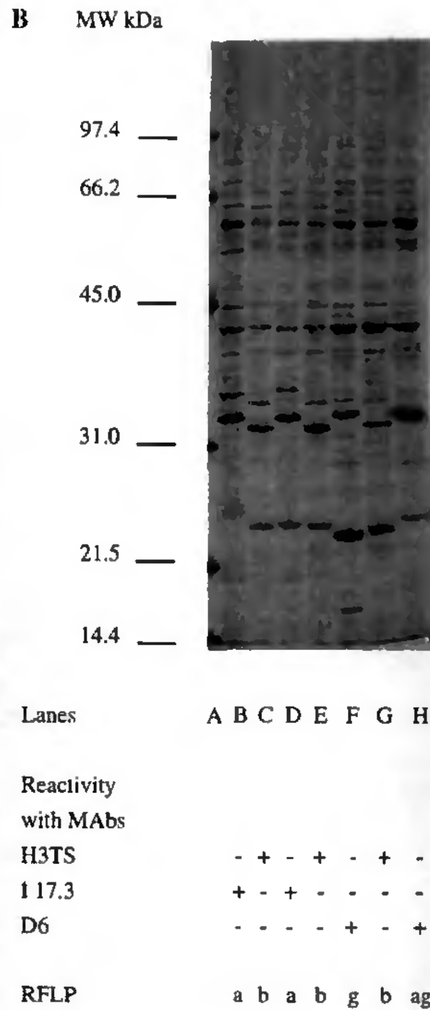

Fig. 3. (*Continued*)

transmission to be maintained efficiently in nature. Vertebrate hosts defined as reservoirs or amplifiers are infected by *B. burgdorferi* sl and act as sources of infection for *I. ricinus* vectors. Practically, the reservoir/amplifier status of a host species should be evaluated using tick xenodiagnosis. This method has been mainly used with animal species which can be easily captured and maintained under laboratory conditions, like *Apodemus* and *Clethrionomys* rodents (Aeschlimann et al., 1986; De Boer et al., 1993; Humair et al., 1993; Kurtenbach et al., 1995). An alternative method consists in comparing infection rate of host-feeding larvae with infection rate of free-living larvae. Initially, the present study aimed to test red squirrels by tick xenodiagnosis. Trappings of squirrels were done but were given up after several attempts in various sites near Neuchâtel, where these animals were

observed. Consequently, we decided to focus on the analysis of dead individuals and attached ticks.

The isolation of *B. burgdorferi* sl from squirrel skin necropsies demonstrates that this animal species is exposed to spirochetal infection as grey squirrels (*S. carolinensis*) in the UK (Craine et al., 1997). In the present study, living spirochetes were obtained from the skin of 4/6 *S. vulgaris*, showing a relatively high prevalence of infection (67%). Borreliae were also present in one squirrel living at high altitude where *I. ricinus* is less frequently found (> 1000 m) (Aeschlimann, 1972), suggesting that a low level of tick population is not a limiting factor for *B. burgdorferi* sl survival.

The prevalence of infection in ticks attached to squirrels varied from 3.3 to 69%. The low number of larvae inoculated in BSKII medium (due to a high mortality rate among larvae between collection and examination) does not allow us to determine the reservoir status of each squirrel, except for squirrel No. 5. Prevalences of infection in *I. ricinus* larvae (64.3%) and nymphs (69.5%), feeding on this host, were far higher than those usually observed in free-living larvae (0–3%, Zhioua et al., 1994) and nymphs (5–34%, Aeschlimann et al., 1986) in Switzerland. Thus, *B. burgdorferi* sl transmission occurs from *S. vulgaris* to feeding ticks. However, we are not able to elucidate whether *S. vulgaris* is a real amplifying/reservoir host or if squirrel-feeding ticks acquired infection via co-feeding transmission (Kimura et al., 1995; Gern and Rais, 1996; Randolph et al., 1996; Sato and Nakao, 1997). This is particularly relevant for animal No. 5 on which ticks were distributed in clusters. However, the demonstration made by Craine et al. (1997) of reservoir competence of *S. carolinensis*, a host species closely related to *S. vulgaris*, led to the supposition that borrelial transmission other than cofeeding transmission also occurs between *S. vulgaris* and attached ticks.

Considering the high tick infestation ($n = 747$) of squirrel No. 5 and the high prevalence of infection in feeding ticks (69%), we can estimate that this individual would have released about 500 infected *I. ricinus* (237 infected larvae and 262 infected nymphs) in its habitat. Even if these data are time-limited data, they show that a single squirrel may transmit *B. burgdorferi* sl to a large number of ticks, particularly during larval and nymphal peak activity. The reservoir competence of a host species is actually defined by three main factors: high infectivity to the vector, high infestation with the vector and host abundance (Mather et al., 1989). Although our data do not allow to evaluate the reservoir competence of squirrels (host abundance unknown), they show that red squirrels can contribute to the maintenance of *B. burgdorferi* sl in nature, considering the high infectivity to ticks and the high infestation by ticks.

B. burgdorferi ss, *B. afzelii*, *B. garinii* and *Borrelia* VS116 are ubiquitous in host-seeking tick populations on the Swiss Plateau (Hu et al., 1994; Leuba-Garcia et al., 1994; Humair et al., 1995; Humair et al., in press). Our data concerning infection in host-seeking ticks presented herein confirmed these observations. That means that in endemic areas in Switzerland animal hosts are in contact with ticks infected by various genospecies. Three genospecies were identified in ticks feeding on squirrels. *B. burgdorferi* ss and *B. afzelii* clearly dominated, whereas *B. garinii*

was less frequently observed. The infection in ticks collected from hosts may come: (1) From a previous infectious blood meal; or (2) from the current blood meal. In the endemic area where squirrels Nos. 1, 2 and 5 were collected, *B.* garinii was often isolated from field-collected ticks (in the present study; Hu et al., 1994). Nevertheless, the high prevalence of *B. burgdorferi* and *B. afzelii* infection in squirrel-feeding ticks suggests that these two genospecies are transmitted from squirrels to feeding ticks rather than the other way round. This is confirmed by the fact that only two genospecies, *B. burgdorferi* ss and *B. afzelii* were isolated from skin samples of squirrels, whereas *B. garinii* was only PCR detected, suggesting that *B. garinii* may have difficulty in establishing infection in squirrels. We suggest that squirrels act as filters and are reservoirs for *B. burgdorferi* ss and *B. afzelii.* Interestingly, this is in accordance with results obtained with grey squirrels in the UK, where *B. afzelii* was observed in the skin of *S. carolinensis* and where successful infection of grey squirrel by NE550 (*B. burgdorferi* ss, Hu et al., 1994) was experimentally obtained (Craine et al., 1997). These observations emphasized the hypothesis made by Nakao et al. (1994) and Humair et al. (1995), who suggested possible associations between *Borrelia* species and host species. Such associations between hosts and genospecies have been shown in Switzerland, where *B. afzelii* was associated with *Apodemus* and *Clethrionomys* rodents (Humair et al., 1995; Hu et al., 1997), and *B. garinii* and *Borrelia* VS116 with *Turdus* passerines (Humair et al., in press). Thus, in the ecology of Lyme borreliosis, vertebrate reservoirs act as biological filters by selecting *Borrelia* species compatible with host physiological environment and then transmit this (these) genospecies to following feeding ticks.

This paper represents a further step towards a better understanding of tick-host-*Borrelia* species relationships by showing an additional association between *Borrelia* genospecies and host species, in this case *B. burgdorferi* ss, *B. afzelii* and red squirrels.

Acknowledgements

This research was supported by the Swiss National Science Foundation (No. 32-29964-90), the Swiss Federal Office of Education and Science (No. 93.0363), the British Council/Swiss National Foundation Joint Research Programme and the European Union Biomedical and Health Research (Biomed I) Programme (No. BMH-CT93-1183). We thank Noel Craine for introducing the squirrel trappings, Katy Sell, Sandrine Meyer and Martine Siegenthaler for providing some of the squirrels, Raquel Martinez and Olivier Rais for their excellent laboratory assistance, Guy Baranton, Alan Barbour and Olivier Péter for the monoclonal antibodies. This work is part of the PhD dissertation of P.F.H.

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Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance

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(*Received 10 April 1998; revised 3 July and 20 July 1998; accepted 20 July 1998*)

SUMMARY

This study deals with the ecology of Lyme borreliosis in Europe. The relationships between *Borrelia burgdorferi sensu lato* spirochetes, *Clethrionomys* and *Apodemus* rodent reservoirs and the *Ixodes ricinus* tick vector were investigated during 16 consecutive months in an enzootic area in Switzerland. Cultivation of ear skin biopsies was used to isolate spirochetes from *C. glareolus*, *A. sylvaticus*, *A. flavicollis* and *Glis glis*. *Borrelia* infection was more frequently observed in *Clethrionomys* than in *Apodemus*. Tick xenodiagnosis was used to determine the infectivity of rodents. The infection rate in ticks fed on *Clethrionomys* was higher than that in ticks fed on *Apodemus*, but *Apodemus* yielded more infected ticks than *Clethrionomys* because of a better tick moulting success. Xenodiagnostic ticks were placed into BSK medium to obtain isolates. Isolates from rodents and rodent-feeding ticks were all identified as *B. afzelii*. The follow-up of the infectivity status of repeatedly recaptured rodents clearly demonstrated that these hosts remained infective for ticks during winter till the following spring. Comparing *C. glareolus* and *A. sylvaticus*, each rodent species showed different host infection, different host infectivity and contributed differently to the moulting success of feeding ticks. These factors influence differentially the pattern of transmission of *B. afzelii* from *Clethrionomys* voles and *Apodemus* mice to *I. ricinus* ticks.

Key words: Lyme borreliosis, ecology, reservoirs, rodents, *Borrelia afzelii*, overwintering.

INTRODUCTION

Lyme borreliosis is a tick-borne disease caused by the spirochete *Borrelia burgdorferi sensu lato* (s.l.) which is commonly transmitted in Europe by the bite of *Ixodes ricinus* ticks. Studies on the ecology of Lyme borreliosis have demonstrated that the efficient persistence of *Borrelia* pathogens in endemic areas requires the involvement of reservoir hosts. Small-sized mammals, *Apodemus* mice and *Clethrionomys* voles in particular, have been studied in various enzootic areas in Europe (Aeschlimann *et al.* 1986; Matuschka *et al.* 1992; de Boer *et al.* 1993; Humair *et al.* 1993; Tälleklint & Jaenson, 1994; Kurtenbach *et al.* 1995). Competent reservoirs were also found among medium-sized mammals – dormice, hedgehogs, rats, squirrels, hares (Gray *et al.* 1994; Matuschka *et al.* 1994, 1997; Tälleklint & Jaenson, 1994; Craine *et al.* 1997; Gern *et al.* 1997; Humair & Gern, 1998) – and ground-frequenting birds – passerines and pheasant (Olsén, Jaenson & Bergström, 1995; Humair *et al.* 1998; Kurtenbach *et al.* 1998*a*). On the other hand, large-sized mammals – moose and deer – are apparently incompetent as reservoirs (Gray *et al.* 1992, 1995; Jaenson & Tälleklint, 1992; Tälleklint & Jaenson, 1994).

The heterogeneity of European *Borrelia* isolates, widely documented since 1986 in free-living ticks of various enzootic areas (Wilske *et al.* 1986) has led to the splitting of *B. burgdorferi* into diverse genospecies. In Europe, 3 pathogenic genospecies belong to this complex: *B. burgdorferi sensu stricto* (s.s.), *B. afzelii* and *B. garinii* (Johnson *et al.* 1984; Baranton *et al.* 1992; Canica *et al.* 1993). Two newly named species apparently non-pathogenic for humans are also included in this complex: *B. valaisiana* (previously group VS116) and *B. lusitaniae* (previously group PotiB2) (Le Fleche *et al.* 1997; Wang *et al.* 1997). Different genospecies generally co-exist in tick populations in Switzerland (Hu *et al.* 1994; Péter, Bretz & Bee, 1995). Which *Borrelia* species infects which host species and the ticks feeding on them remained enigmatic for a long time, since very few isolates were obtained from hosts (Hovmark *et al.* 1988) and none were obtained from host-feeding ticks until recently (Hu *et al.* 1997). However, specific associations between hosts, ticks and *Borrelia* species were observed: *B. afzelii* and small rodents in Switzerland (Humair *et al.* 1995; Hu *et al.* 1997), *B. burgdorferi* s.s. and small rodents in the UK (Kurtenbach *et al.* 1998*a*), *B. garinii* and

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B. *valaisiana* and birds (Humair *et al.* 1998; Kurtenbach *et al.* 1998*a*), *B. afzelii* and grey squirrels in the UK (Craine *et al.* 1997) and *B. burgdorferi* s.s. and *B. afzelii* and red squirrels in Switzerland (Humair & Gern, 1998).

In this study, we investigated the maintenance of *B. burgdorferi* s.l. in *Clethrionomys* and *Apodemus* rodents over 16 months in an enzootic area in Switzerland. For this purpose, *B. burgdorferi* s.l. was isolated from hosts and from rodent-feeding ticks, and tick xenodiagnosis was used to assess host infectivity for ticks. The identification of *Borrelia* species allowed precise investigation of the association between *Borrelia* and rodent reservoirs.

MATERIALS AND METHODS

Study site

Small mammals and questing ticks were collected at Glütschbachtal near Thun (Canton of Berne, Switzerland, altitude 589 m), at the same enzootic site described previously (Humair *et al.* 1995).

Investigation of small mammals and infesting ticks

Small mammals were trapped alive for 2 successive nights each month from May 1993 to August 1994. One hundred wooden box traps were placed in a grid of 4 lines of 25 traps with identical location for each capture session. Traps were baited with grains, sunflower seeds and pieces of apple. Each small mammal, except shrews, was brought into the laboratory and caged individually over a pan of water until the feeding ticks dropped off. Engorged ticks were collected daily and maintained at relative humidity close to saturation (RH $> 95\%$) and at room temperature until the moult was completed. After about 1 week of captivity, each rodent was anaesthetized, identified to species, sexed, aged (juvenile or adult) and marked with numbered ear-tag. An ear skin biopsy was taken for isolation of *B. burgdorferi* s.l. Tick xenodiagnosis was performed to study infectivity of rodents. Finally, rodents were released at the exact trapping site, generally 14 days after capture. Retrapped animals were identified by marks and were re-examined. Attached ticks were removed by forceps from the single *Glis glis*, an ear skin biopsy was taken, and the individual was released the same day at the capture site.

Tick xenodiagnosis

Xenodiagnosis was performed 1–2 days after all field-derived ticks were detached from hosts. About 40 xenodiagnostic *I. ricinus* larvae, derived from a laboratory colony free of spirochetal infection, were placed on the head of rodents and were allowed to engorge. Replete xenodiagnostic ticks were collected daily from each host into a pan of water and maintained in the same conditions as described for field-derived ticks, until they moulted.

Collection of free-living ticks

Host-seeking *I. ricinus* nymphs and adults were collected by flagging the vegetation at the site of collection of small mammals and in the close vicinity once a month from August to October 1993 and from March to June 1994. Ticks were identified to species, stage and sex, and were maintained as described above for engorged ticks until processed for spirochete isolation.

Isolation of spirochetes

Ear skin biopsies were collected from anaesthetized rodents and from 4 dead shrews to isolate spirochetes. Skin samples (1–2 mm^2) were taken with sterilized scissors after cleaning the ear with 70% ethanol and were immediately placed into tubes containing supplemented BSKII medium as described by Sinsky & Piesman (1989). Tissue samples from liver, heart, spleen, kidneys and brain were removed from 1 *C. glareolus* (No. 168) and also placed into supplemented BSKII medium (Sinsky & Piesman, 1989).

Ticks collected on rodents and free-living ticks were briefly soaked in 70% ethanol and individually squashed with sterilized forceps in tubes containing BSKII medium modified by Sinsky & Piesman (1989).

The above cultures were screened by dark-field microscopy for the presence of spirochetes after 10 days, and 3, 6, and 8 weeks of incubation at 34 °C. Cultivable spirochetes were subcultured to allow analyses by SDS–PAGE and Western blot. Spirochetes in original culture tubes were identified by PCR/RFLP.

SDS–PAGE and Western blot

After 2–4 subcultures, rodent and tick isolates were analysed by SDS–PAGE and Western blots using species-specific monoclonal antibodies allowing *Borrelia* identification, as described elsewhere (Humair *et al.* 1998).

RFLP analysis

Polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) described by Postic *et al.* (1994) were used to identify the *Borrelia* species. The variable intergenic spacer between repeated 23S (*rrl*) – 5S (*rrf*) ribosomal genes of *B. burgdorferi* s.l. was used as a template for PCR and RFLP analysis using *Mse* I endonuclease.

Table 1. Infestation of rodents with larval and nymphal *Ixodes ricinus* (Glütschbachtal, Switzerland, May 1993–August 1994)

Scientific name Common name	No. of hosts examined	No. of hosts infested with larvae* (%)	Larvae Number	Larvae Mean no. per host ± S.D.	No. of hosts infested with nymphs (%)	Nymphs Number	Nymphs Mean no. per host ± S.D.
Apodemus flavicollis Yellow necked mouse	26†	24 (92·3)	143	5·5 ± 4·7	3 (11·5)	5	0·2 ± 0·6
Apodemus sylvaticus Wood mouse	60	37 (61·7)	362	6·0 ± 11·2	8 (13·3)	19	0·3 ± 1·3
Apodemus sp.	3	2 (66·7)	2	0·7 ± 0·6	0 (0)	0	
Clethrionomys glareolus Bank vole	107	79 (73·8)	770	7·2 ± 10·1	13 (12·1)	19	0·2 ± 0·6
Microtus agrestis Field vole	3‡	0 (0)					
Glis glis Edible dormouse	1	1 (100)	5		1 (100)	1	
Total	200	143 (71·5)	1282	6·4 ± 9·8	25 (12·7)	44	0·2 ± 0·8

* Corresponds to the number of hosts with *I. ricinus* subadults, since nymphs were always attached with larvae.
† Captured during tick activity period.
‡ Captured in January and February.

Borrelia DNA was amplified by PCR directly from initial cultures to identify the genospecies present in the original materials and to detect mixed infections. For this procedure, 2 ml were used from cultures that contained spirochetes as observed by dark-field microscopy, whereas the whole volume (4 ml) was used for those cultures that were negative by dark-field microscopy after 8 weeks incubation at 34 °C.

Detection of spirochetes by immunofluorescence

For rodents from which no ticks were cultivated or from which tick cultivation and PCR detection were negative, xenodiagnostic rodent-feeding ticks were also examined by direct immunofluorescence (IF), as described previously (Humair *et al.* 1993).

Statistical analysis

The Fisher's exact test was used to compare the proportions. Probabilities of $P < 0{\cdot}05$ were considered statistically significant.

RESULTS

Investigation of small mammals

Over 3200 trap nights, a total of 214 micromammals of 6 different species were captured at Glütschbachtal from May 1993 to August 1994: 107 *C. glareolus*, 60 *A. sylvaticus*, 26 *A. flavicollis*, 3 *Apodemus* sp., 14 *Sorex araneus*, 3 *Microtus agrestis* and 1 *G. glis*. Thirty-three animals (20 *C. glareolus*, 9 *A. sylvaticus*, 3 *A. flavicollis*, 1 *M. agrestis*) were recaptured once or several times (up to 10 times for 1 individual) during the study period.

Tick infestation of rodents and tick moulting success

Between May 1993 and August 1994, 200 rodents were examined for ticks (shrews were not examined) and 1372 ticks were obtained. *I. ricinus* was the dominant tick species ($n = 1326$, 96·6 %), while the remaining 3·4 % comprised 46 *I. trianguliceps* (36 larvae, 9 nymphs, 1 female) and 1 larva of *I. hexagonus*. Of the attached *I. ricinus*, larvae were far more numerous ($n = 1282$, 96·7 %) than nymphs ($n = 44$, 3·3 %) (Table 1). Mean numbers of ticks per rodent reached 6·4 for larvae and 0·2 for nymphs (Table 1).

Attached *I. ricinus* ticks were found on rodents from May to November 1993 and from April to August 1994, except for 1 larva found on a wood mouse in January 1994 (Fig. 1). During these periods, the prevalence of infested rodents reached more than 90 % for the 3 most abundant rodent species. The monthly median number of larvae per rodent showed a bimodal seasonal pattern in 1993 (Fig. 1), with peaks in May and in August/September. In 1994, the seasonal pattern is incomplete but the spring peak of infestation was also present in May.

The moulting success of field-derived *I. ricinus* ticks fed on *A. sylvaticus*, *A. flavicollis* and *C. glareolus* reached 33·3 % (127/381), 14·9 % (22/148) and 6·2 % (49/789), respectively. The same was observed for xenodiagnostic ticks fed on *A. sylvaticus* (34·2 %, 515/1506), *A. flavicollis* (31·5 %, 115/365) and *C. glareolus* (18·3 %, 234/1280). Interestingly, the proportion of hosts with ticks which moult successfully was higher for *A. sylvaticus* (51/58, 87·9 %) than for *A. flavicollis* (15/25, 60·0 %, $P = 0{\cdot}006$) or for *C. glareolus* (41/94, 43·6 %, $P < 0{\cdot}001$).

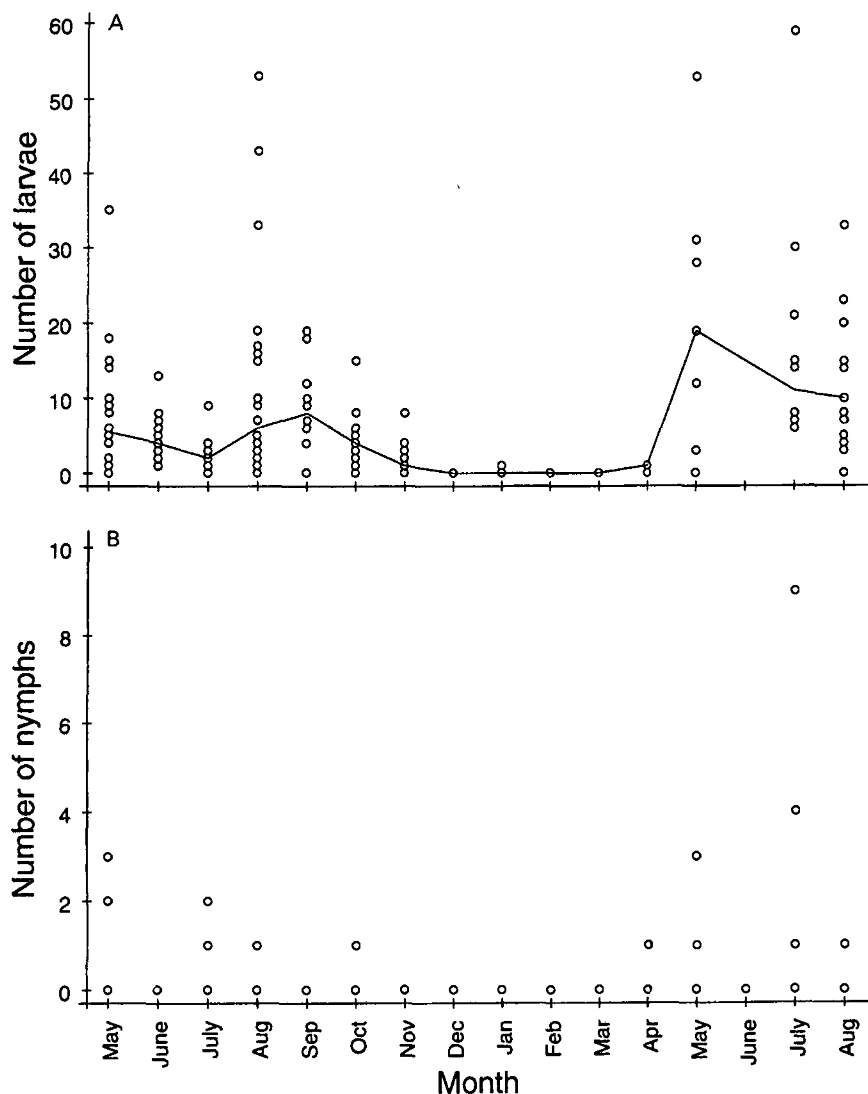

Fig. 1. Seasonal distribution of *Ixodes ricinus* larvae (A) and nymphs (B) on rodents (*Apodemus* and *Clethrionomys*). Seasonal distribution of the monthly median number of larvae per rodent (—). (Glütschbachtal, Switzerland, May 1993–August 1994).

Table 2. Isolation and DNA detection of *Borrelia burgdorferi sensu lato* from rodent ear biopsies (Glütschbachtal, Switzerland, May 1993–August 1994)

Species	No. of hosts examined	Isolation rate (%)	DNA detection rate in negative cultures (%)	Host infection rate* (both methods) (%)
A. flavicollis	26	1/29† (3·4)	0/6§ (0)	1/26 (3·8)
A. sylvaticus	60	4/63† (6·3)	1/13¶ (7·7)	5/60 (8·3)
Apodemus sp.	3	0/3 (0)	0/1 (0)	0/3 (0)
C. glareolus	98	25/106‡ (23·6)	5/15‖ (33·3)	27/98 (27·6)
M. agrestis	3	0/3 (0)	—	0/3 (0)
G. glis	1	1/1 (100)	—	1/1 (100)
S. araneus	4	0/4 (0)	—	0/4 (0)
Total	195	31/209 (14·8)	6/35 (17·1)	34/195 (17·4)

* No. of infected hosts/No. of hosts examined.
† For 3 individuals, 2 skin biopsies were taken.
‡ For 8 individuals, 2 skin biopsies were taken.
§ From 3 individuals.
¶ From 10 individuals.
‖ From 12 individuals.

Table 3. Success of tick xenodiagnosis on field-collected rodents evaluated through immunofluorescence, isolation and DNA detection of *Borrelia burgdorferi sensu lato* in cultures of xenodiagnostic *Ixodes ricinus* ticks

	Hosts	Xenodiagnostic ticks			
Species	No. of hosts with positive xenodiagnosis* (%)	Isolation rate (%)	DNA detection in negative culture tubes (%)	Detection rate by immunofluorescence (%)	Tick infection rate (all methods) (%)
A. flavicollis	1/10 (10·0)	0/61 (0)	2/55 (3·6)	0/31 (0)	2/92 (2·2)
A. sylvaticus	22/50 (44·0)	22/244 (9·0)	45/182 (24·7)	9/75 (12·0)	76/319 (23·8)
Apodemus sp.	0/2 (0)	0/3 (0)	0/3 (0)	0/1 (0)	0/4 (0)
C. glareolus	19/37 (51·4)	6/110 (5·5)	29/92 (31·5)	15/39 (38·5)	50/149 (33·6)
M. agrestis	0/2 (0)	0/11 (0)	0/11 (0)	0/8 (0)	0/119 (0)
Total	42/101 (41·6)	28/429 (6·5)	76/343 (22·2)	24/154 (15·6)	128/683 (18·7)

* Obtained by all methods.

Isolation and DNA detection of B. burgdorferi *s.l. from mammal tissues*

A total of 209 ear skin biopsies were removed from 195 small mammals (for 14 individuals, 2 skin biopsies were taken) for the isolation of spirochetes (Table 2). Thirty-one isolates were obtained from 29 animals of 4 rodent species (*C. glareolus*, *A. sylvaticus*, *A. flavicollis* and *G. glis*). One additional isolate was obtained from a heart sample from 1 *C. glareolus*, from which no spirochete was isolated from left or right ear biopsies. All isolates, except 2 which could not be amplified by PCR, were analysed by RFLP and identified as *B. afzelii*. Protein profiles of 21 isolates, including the heart isolate, presented a protein profile typical of *B. afzelii* with an OspA of 32 kDa and an OspB of 35 kDa, and all isolates reacted to MAb I.17.3 specific for *B. afzelii*.

In addition, 35 initial culture tubes containing ear biopsies of 26 small mammals trapped from April to August 1994 and which remained negative under dark-field microscopy, were screened by PCR (Table 2). *Borrelia* DNA was detected in 6 culture tubes of 5 additional rodents (4 *C. glareolus* and 1 *A. sylvaticus*). *B. afzelii* was detected in 5 cases and 1 mixed infection of *B. afzelii* and *B. burgdorferi* s.s. was observed in 1 *C. glareolus* trapped in August 1994.

The infection rate of *C. glareolus*, evaluated by isolation and DNA detection of *B. burgdorferi* s.l. from mammal tissues, reached 27·6 % (Table 2). This rate was significantly higher than those of *A. sylvaticus* (8·3 %, $P = 0{\cdot}004$) and *A. flavicollis* (3·8 %, $P = 0{\cdot}015$).

Detection of B. burgdorferi *s.l. in field-derived rodent-feeding ticks*

The cultivation of 77 *I. ricinus* ticks (70 nymphs and 7 adults) – attached on captured rodents as larvae and nymphs – yielded 17 isolates: 13 from nymphs (*A. sylvaticus*: 12/37, *A. flavicollis*: 1/9, *C. glareolus*: 0/22) and 4 from adults (*A. sylvaticus*: 3/6, *C. glareolus*: 1/3). RFLP, SDS–PAGE and Western blot revealed that all isolates belonged to *B. afzelii*. Three out of 4 *I. ricinus* larvae partly-engorged on *G. glis* were found to be positive by IF.

Detection of B. burgdorferi *s.l. in xenodiagnostic ticks (isolation, DNA detection and IF).*

A total of 683 xenodiagnostic ticks collected from 101 rodents were examined either by *Borrelia* isolation, DNA detection or IF. The cultivation of 429 xenodiagnostic *I. ricinus* nymphs fed on 79 rodents as larvae yielded a total of 28 isolates (Table 3). All these isolates, except 2 which could not be amplified by PCR, were identified as *B. afzelii* by RFLP analysis. Isolates tested by SDS–PAGE and Western blot showed complete correspondence with RFLP results.

A PCR screening was used to detect *Borrelia* DNA in 343 initial culture tubes which remained negative in dark-field microscopy. *Borrelia* DNA was detected in 76 culture tubes containing xenodiagnostic ticks, mostly those that had fed on *C. glareolus* and *A. sylvaticus* (Table 3). DNA of *B. afzelii* ($n = 73$) and *B. burgdorferi* s.s. ($n = 2$) was detected. One mixed infection (*B. afzelii*/*B. burgdorferi* s.s.) was observed.

Using IF, infected ticks (24/154) were obtained from 6 additional *C. glareolus* and 4 additional *A. sylvaticus* (Table 3).

To sum up, positive xenodiagnoses (isolation, DNA detection and IF) were obtained from *C. glareolus*, *A. sylvaticus* and *A. flavicollis* (Table 3). Many more *C. glareolus* (51·4 %) and *A. sylvaticus* (44·0 %) were infective for ticks than *A. flavicollis* (10·0 %, $P = 0{\cdot}029$ and $P = 0{\cdot}073$, respectively). The infection rate in ticks fed on *C. glareolus* (33·6 %) was higher than those in ticks fed on *A. sylvaticus*

Animal N°	1993 Jun	July	Aug	Sept	Oct	Nov	Dec	1994 Jan	Feb	Mar	Apr	May	Jun	July	Transmission coefficient [1]
Apodemus sylvaticus															
42 A B			0/1		1/3		1/1	6/11	0/3	6/12		3/10*			0.415
124 A B							3/3	2/6	2/3	6/10					0.590
125 A B						0/1	1/4	2/3	4/9	3/6	3/3	8/10		3/3	0.615
146 A B								3/9	4/10	0/13					0.219
Clethrionomys glareolus															
26◇ A B		2/2		# 1/1	1/1			1/1							1.000
36 A B		4/15			0/1										0.250
48 A B					0/1										0.000
54 A B					0/1		1/3								0.250
110 A B							8/10	4/10	2/3						0.609
115 A B							2/2								1.000
159 A B										6/9	3/5	3/5†		2/3	0.636
	WILD TICKS ON HOSTS						NO WILD TICK ON HOSTS				WILD TICKS ON HOSTS				

A. Cultivation of skin biopsies
- positive
- negative
- not done

B. Xenodiagnosis
- positive
- negative
- ticks did not moult
- not done

Fig. 2. Follow-up of host infection using skin biopsy cultivation and host infectivity using tick xenodiagnosis of infected *Apodemus sylvaticus* mice and *Clethrionomys glareolus* voles repeatedly recaptured during winter (November 1993–March 1994). [1] Transmission coefficient is the product of the probability of an infected host passing the infection to a tick and the probability of the infection being maintained transstadially from engorged larva to unfed nymph (Randolph & Craine 1995). * One xenodiagnostic tick presented mixed infection with *B. afzelii* and *B. burgdorferi sensu stricto*. ◇ Animal No. 26 was kept in captivity after its first recapture in August 1993. Since then, the animal had no contact with potentially infective ticks and remained infective for ticks. # Positive blood culture and negative skin biopsy culture. † One xenodiagnostic tick was infected by *B. burgdorferi sensu stricto*.

(23·8 %, $P = 0{\cdot}033$) and on *A. flavicollis* (2·2 %, $P < 0{\cdot}001$). Numerically, however, *A. sylvaticus* yielded more infected ticks than *C. glareolus* because of a better tick moulting success after feeding on *Apodemus* mice. Tick xenodiagnosis was a more efficient method of revealing borrelial infection in *C. glareolus* (51·4 %, $P = 0{\cdot}014$) and in *A. sylvaticus* (44·0 %, $P < 0{\cdot}001$) than was cultivation of ear biopsy (27·6 % and 8·3 %, respectively).

Comparing the infectivity of rodents to xenodiagnostic ticks with their infection status (using *Borrelia* isolation), we observed that only very few *A. sylvaticus* infective for ticks also yielded spirochetes isolated by skin biopsy cultivation (4/22, 18 %), whereas about half the infective *C. glareolus* gave positive skin spirochetal infection (10/19, 53 %).

From our data, the transmission coefficient from vertebrate hosts to ticks (Randolph & Craine, 1995) can be measured to assess the infectivity of each rodent species. The transmission coefficient reached 0·653 for *C. glareolus*, 0·542 for *A. sylvaticus* and 0·217 for *A. flavicollis*. The differential tick survival after feeding on specific hosts may, however, modify the relative contribution of each rodent species as reservoirs (see the Discussion below).

Isolation of B. burgdorferi *s.l. from free-living ticks*

From 235 free-living *I. ricinus* ticks (93 nymphs, 69 females, 73 males) collected by flagging, a total of 45 isolates were obtained: 8 from nymphs, 19 from females and 18 from males. The RFLP identification revealed 18 *B. burgdorferi* s.s., 11 *B. afzelii*, 7 *B. garinii* and 2 unidentified *Borrelia*. Two double (*B. burgdorferi* s.s. and *B. afzelii*) and 1 triple (*B. burgdorferi* s.s., *B. afzelii* and *B. garinii*) mixed infections were observed, but only in adult ticks. Ten isolates (9 *B. burgdorferi* s.s and 1 *B. afzelii*) presented atypical RFLP profiles. DNA of 4 isolates were not amplified. Isolates analysed by SDS–PAGE showed typical protein profiles, except for some isolates which presented atypical electrophoretic mobilities of OspA and OspB. Complete correspondence was observed between reaction to MAbs and respective species identification by RFLP.

Repeated observations of infection and infectivity of rodents captured during winter

The changing infection status, using *Borrelia* isolation, and the infectivity status, using tick xenodiagnosis, of infected rodents repeatedly recaptured during winter (November to March) is presented in Fig. 2. *A. sylvaticus* and *C. glareolus* remained infective for ticks during winter till the following spring and the individual infectivity (Mather *et al.* 1989) or coefficient of transmission (Randolph & Craine, 1995) of rodents varied between 0·219 and 1·000 (Fig. 2). Infection using *Borrelia* isolation from skin biopsy is more easily detected in *C. glareolus* than in *A. sylvaticus* as already observed above. Fig. 2 clearly shows the 'negative cultivation/positive xenodiagnosis' pattern characterizing *A. sylvaticus*, and the 'positive cultivation/positive xenodiagnosis' pattern as well as the 'positive cultivation/infectivity not determined' pattern due to failure of tick moult characteristic of *C. glareolus*.

DISCUSSION

This study supports previous reports on the importance of *Apodemus* mice and *Clethrionomys* voles in the ecology of *I ricinus* subadults and of *B. burgdorferi* s.l., and it highlights the differential transmission pattern of *B. burgdorferi* s.l. between these hosts and ticks.

The tick infestation of *C. glareolus*, *A. sylvaticus* and *A. flavicollis* observed at Glütschbachtal (May–October 1993) was different from that observed at Staatswald (May–October 1988) 50 km away (Humair *et al.* 1993). The prevalence of tick infestation on *C. glareolus* was higher at Glütschbachtal (90·0 %) than at Staatswald (77·6 %, $P = 0{\cdot}048$) as was the mean number of larvae per *C. glareolus* ($P = 0{\cdot}054$). Using tick xenodiagnosis, the infectivity of *C. glareolus* was twice as high at Glütschbachtal (51·4 %) as at Staatswald (20·5 %, $P = 0{\cdot}005$). The dominance of *C. glareolus* as the primary reservoir of *B. burgdorferi* s.l. at Glütschbachtal in 1993–94 corresponds to the situations observed in foci in Sweden in 1991 and 1992 (Tälleklint & Jaenson, 1994) and in Germany in 1988 and 1990 (Kurtenbach *et al.* 1995), but contrasts with the situation observed in 1988 at Staatswald, where *Apodemus* mice played the dominant reservoir role (Humair *et al.* 1993). In Europe, the respective contribution of *C. glareolus*, *A. sylvaticus* and *A. flavicollis* as hosts for ticks and as reservoirs for the Lyme borreliosis spirochetes varies geographically and temporally (seasonally and from year to year). The ratio of *I. trianguliceps*/*I. ricinus* ticks attached to rodents was greater at Glütschbachtal in 1980–82 (1:4, Gern & Aeschlimann, 1986) than in 1993–94 (1:29, $P < 0{\cdot}001$) showing that the proportion of *I. trianguliceps*/*I. ricinus* ticks can change markedly in a biotope in a decade.

In the studies of Tälleklint & Jaenson (1994) and Kurtenbach *et al.* (1995), demonstrating the importance of *C. glareolus* in Sweden and in Germany, the relative contribution of rodents was assessed according to the reservoir potential developed by Mather *et al.* (1989). The reservoir potential (Mather *et al.* 1989) is defined by the following formula: $R_s = I_s L_s D_s / \Sigma_s (I_s L_s D_s)$, where I_s is the proportion of larvae that become infected after feeding on a host species (s), L_s is the average number of larvae infesting that host species, and D_s is the local host density. In the present study, the reservoir potential reached 0·745 for *C. glareolus* ($I_s = 0{\cdot}336$, $L_s = 7{\cdot}2$, $D_s = 7{\cdot}3$), 0·247 for *A. sylvaticus* ($I_s = 0{\cdot}238$, $L_s = 6{\cdot}0$, $D_s = 4{\cdot}1$) and 0·008 for *A. flavicollis* ($I_s = 0{\cdot}022$, $L_s = 5{\cdot}5$, $D_s = 1{\cdot}6$). This clearly demonstrated as well the dominance of *C. glareolus* as the main rodent reservoir. But this formula does not take into account the tick moulting success, which is dependent on the biology of the host species on which ticks feed. Dizij & Kurtenbach (1995) have shown, however, in an experimental study in the laboratory that the moulting success of ticks fed on *Clethrionomys* was lower than that of ticks fed on *Apodemus*. In the present study, we observed the same phenomenon with ticks fed on wild hosts, both for field-derived ticks and for xenodiagnostic ticks. This point should be taken into account to evaluate as accurately as possible the relative contribution of a host species as reservoir for *B. burgdorferi* s.l. in a particular enzootic site. The tick survival of xenodiagnostic ticks fed on *C. glareolus*, *A. sylvaticus* and *A. flavicollis* reached 0·183, 0·342 and 0·315, respectively. The framework developed by Randolph & Craine (1995) takes into account this parameter. The

product of the vector/host ratio, the vertebrate-to-tick transmission coefficient, and the tick's inter-stadial proportional survival give an estimate of the relative R_0 index for each host species. This gave a value of 0·860 for *C. glareolus*, 1·112 for *A. sylvaticus* and 0·376 for *A. flavicollis*. To obtain the full R_0 indices, the duration of infectivity and the transmission coefficient from ticks to vertebrate hosts should also be considered, as exposed by Randolph, Gern & Nuttall (1996), but more data concerning these parameters should be obtained to have better estimates. Anyway, considering the moulting success of feeding ticks, the relative contribution of *A. sylvaticus* as reservoirs is higher than that of *C. glareolus*, despite the high infection rate of *C. glareolus*.

We observed that the borrelial infection was more easily detectable from *Clethrionomys* voles than from *Apodemus* mice using cultivation of ear skin biopsy, whereas the prevalence of infection was almost equivalent for *C. glareolus* and *A. sylvaticus* using tick xenodiagnosis. This discrepancy might be caused by the number of spirochetes present in the skin samples and this phenomenon may be related to the stronger immune response to *B. burgdorferi* s.l. in *Apodemus* mice than in *Clethrionomys* voles (Kurtenbach *et al.* 1995). Thus, the inability to isolate *Borrelia* from *Apodemus* skin could be explained either by a control of the number of spirochetes by the host immune response or by the fact that they invaded other organs than skin. The immunosuppressive properties of tick saliva (Ribeiro *et al.* 1985) acting locally could help the ingestion of spirochetes by the feeding ticks. It is known that the continuous exposure of infected rodents to tick bites enhances the transmission of spirochetes from rodents to ticks (Gern *et al.* 1994).

The overwintering maintenance of *B. burgdorferi* s.l. has been ascribed to ticks rather than rodent reservoirs (de Boer *et al.* 1993; Tälleklint & Jaenson, 1995). It is known that infected ticks maintain the pathogen during starvation. However, our observations clearly show that *C. glareolus* and *A. sylvaticus* also act as overwintering reservoirs of *B. afzelii* since they remain infective to ticks during winter. Once infected, small rodents remain infective to ticks for life (Gern *et al.* 1994), although infectivity may vary temporally. In our view, ticks and rodents contribute in their own way to the maintenance of *B. burgdorferi* s.l. from one year to the next in enzootic areas. Overwintering infected rodents are infective to ticks directly at the onset of tick activity. On the other hand, overwintering infected ticks will infect hosts at once, before infected ticks produced later in the season by the overwintering infected rodents. This phenomenon probably permits a more efficient maintenance of the pathogen in the focus, yielding infected ticks and hosts throughout the season of tick activity.

Our results provide yet more strong evidence of the infection of *Clethrionomys* voles and *Apodemus* mice with *B. afzelii* (Humair *et al.* 1995) and the transmission of *B. afzelii* from rodents to ticks (Hu *et al.* 1997), in a second Swiss enzootic focus where various *Borrelia* genospecies occur. Neither *B. burgdorferi* s.s. nor *B. garinii* nor *B. valaisiana* were ever isolated from rodent skin or from rodent-feeding ticks. Only DNA of *B. burgdorferi* s.s. was amplified by PCR in 1 rodent skin and in 3 feeding ticks but this *Borrelia* species was never isolated, suggesting that the infection with this genospecies may occur in the rodents but certainly at too low a level to be efficiently transmissible to ticks. Moreover, the fact that *B. burgdorferi* s.s. was observed only during the period of tick activity on hosts, but not during winter, suggests that *B. burgdorferi* s.s. infection is short-lived in mice and voles. The association between mice and voles and *B. afzelii* is very specific and is not the consequence of a selection process due to the cultivation, since similar associations involving passerines, *B. valaisiana* and *B. garinii*, or squirrels, *B. burgdorferi* s.s. and *B. afzelii* have also been demonstrated using the same methods (Humair & Gern, 1998; Humair *et al.* 1998).

The immune system of vertebrate hosts living in a focus of Lyme borreliosis is confronted with repeated tick bites and with *Borrelia*. Comparing *Apodemus* and *Clethrionomys* immunity, each host species seems to have developed a different strategy towards tick infestation and *Borrelia* infection. *Apodemus* mice do not acquire a resistance to ticks but develop a stronger immune response to *Borrelia* infection (Kurtenbach *et al.* 1994; Dizij & Kurtenbach, 1995). *Clethrionomys* voles react immunologically the other way round. Our observations suggest that, through their immune system, *Apodemus* maintains the borrelial infection at a low level, rarely detectable by skin cultivation, but enough to infect ticks efficiently. On the other hand, *Clethrionomys* voles develop an immune response primarily to ticks, while allowing borreliae to multiply in the host skin. Spirochetes are easily detectable in *Clethrionomys* and are transmitted to ticks. Vole-fed ticks, however, do not engorge or moult successfully because of the acquired resistance of *Clethrionomys* voles to ticks (Dizij & Kurtenbach, 1995; our own data). This indicates that the reservoir competence of a host is modulated by its immune response to the pathogen and to the vector. Similarly, the host immune system probably reacts differentially to the different *Borrelia* genospecies and this could explain the host–*Borrelia* associations already observed (Kurtenbach *et al.* 1998*b*).

The immunological aspects of reservoir hosts should be particularly investigated in the future to understand how reservoirs work, why associations exist between some groups of hosts and *Borrelia*

genospecies, why hedgehogs can be infected by several genospecies (Gern *et al.* 1997) and why large-sized mammals like deer are incompetent as reservoirs (Jaenson & Tälleklint, 1992).

This research was supported by the Swiss National Science Foundation (No. 32-29964-90), the Swiss Federal Office of Education and Science (No. 93.0363), the British Council/Swiss National Foundation Joint Research Programme, and the European Union Biomedical and Health Research (Biomed 1) Programme (No. BMH-CT93-1183). We thank Sarah Randolph for helpful comments and suggestions for improvement of the manuscript. We thank Danièle Postic for the identification of isolates showing atypical RFLP profiles, Guy Baranton and Olivier Péter for the monoclonal antibodies and Jacqueline Moret for statistical analysis. This work is part of the Ph.D. dissertation of P.F.H.

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