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

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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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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

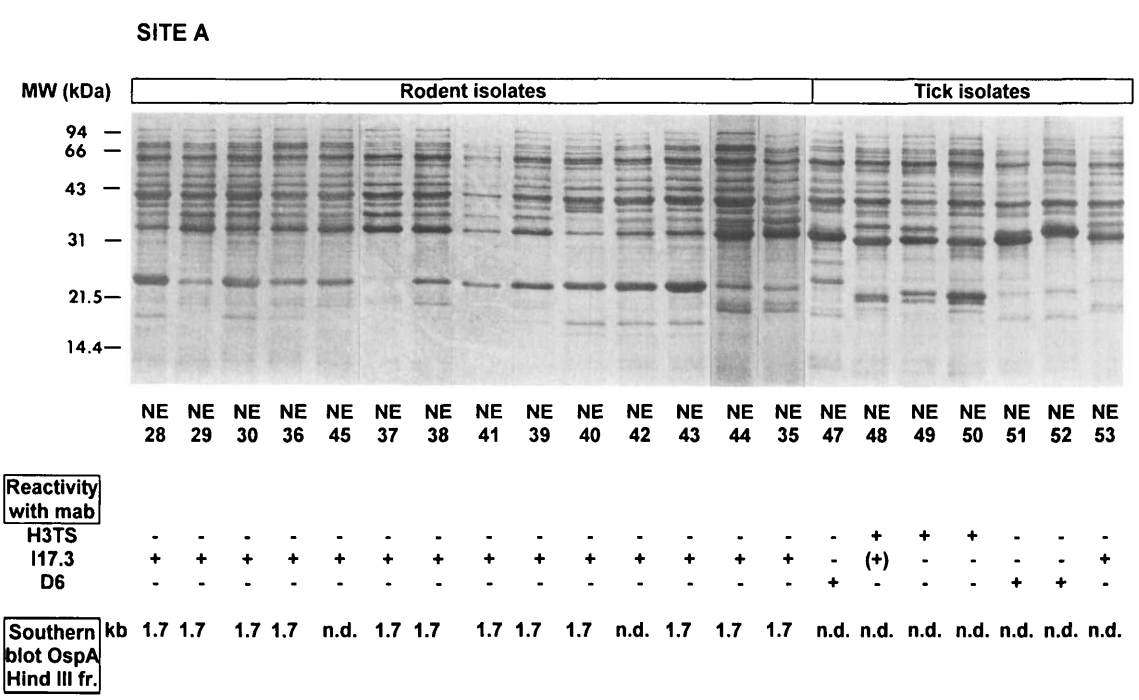

Fig. 1. Protein profiles of *Borrelia* isolates from rodents and ticks from site A. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi sensu stricto*), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). Southern blotting: size (kb) of *Hind*III fragment for the *ospA* 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

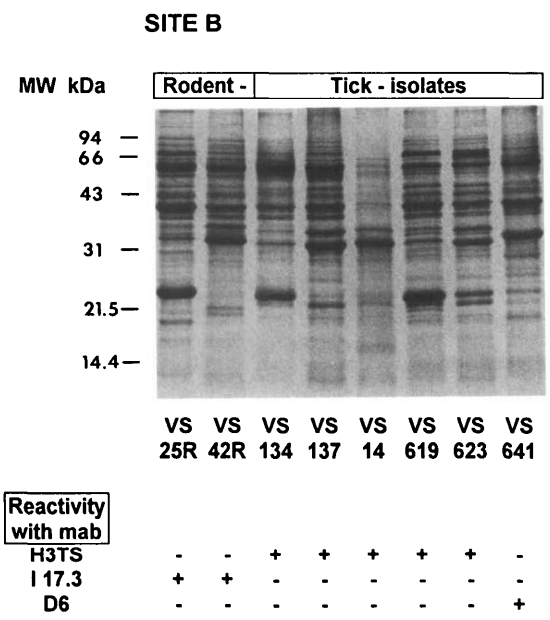

Fig. 2. Protein profiles of *Borrelia* isolates from rodents and ticks from site B. Immunoblotting: reactivity with monoclonal antibodies H3TS (anti-*B. burgdorferi sensu stricto*), I 17.3 (anti-*B. afzelii*), and D6 (anti-*B. garinii*). MW, molecular weight standard; VS 25R, isolate from *A. flavicollis* 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.

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