

Isolation of a Cultivable Spirochete from *Ixodes ricinus* Ticks of Switzerland

Alan G. Barbour,* Willy Burgdorfer,** Stanley F. Hayes,† Olivier Péter,** and André Aeschlimann§

*Laboratory of Microbial Structure and Function. National Institutes of Health. National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840, USA

**Epidemiology Branch, Rocky Mountain Laboratories

†Operations Branch, Rocky Mountain Laboratories

§Zoological Institute, University of Neuchâtel, Neuchâtel 2000, Switzerland

Abstract. We have isolated in BSK medium a spirochete of *Ixodes ricinus* ticks. The ticks were collected from an area of Switzerland where erythema chronicum migrans, a tick-borne, penicillin-ameliorated inflammatory disorder, is endemic. The *I. ricinus* spirochete was very similar in its morphology, polyacrylamide-gel-electrophoresis profile, and antigenic determinants to a spirochete that was previously isolated from *Ixodes dammini* ticks of the United States.

Erythema chronicum migrans (ECM), an expanding annular skin lesion, was first observed in Europe by Afzelius in 1908 [1]. The ixodid tick, *Ixodes ricinus*, has been implicated as a vector for transmission of ECM in Europe [5,15]. ECM is also a common finding in Lyme disease, a penicillin-ameliorated inflammatory disorder of the skin, joints, nervous system, and heart [10,11]. Lyme disease and ECM have only recently been described in the United States [8,12] and, like ECM in Europe, are associated with a preceding bite by an ixodid tick, in this case, *Ixodes dammini* [9,16].

We have previously reported the isolation and cultivation of a spirochete from *I. dammini* ticks collected from Shelter Island, NY, where Lyme disease is endemic [3]. Lyme disease patients from Shelter Island had antibodies to the *I. dammini* spirochete (IDS) during convalescence or the chronic phases of the disorder [3]. The occurrence of ECM and Lyme disease in western Switzerland [6; unpublished observations, A. Aeschlimann] prompted our examination of the implicated vector, *I. ricinus*, from this area. Spirochetes were seen in approximately 30% of *I. ricinus* ticks collected from a forest in western Switzerland [4]. We report here the isolation from infected *I. ricinus* ticks of a spirochete that has many of the features of the IDS.

Materials and Methods

BSK medium was derived from the "fortified Kelly's medium" described by Stoenner et al. [14]. All constituents were in glass distilled water (Corning Glass Works, Corning, NY, no. MP-12A). We combined 100 ml of 10X CMRL 1066 with glutamine (Gibco Laboratories, Grand Island, NY, no. 330-1545) and 900 ml of water. We then added in the following order: 5 g neopeptone (Difco Laboratories, Detroit, MI); 50 g bovine serum albumin, fraction V (BSA, Miles Laboratories, Elkhart, IN, no. 81-003); 6.0 g N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma Chemical Co., St. Louis, MO); 0.7 g sodium citrate; 5 g glucose; 0.8 g sodium pyruvate; 0.4 g N-acetylglucosamine (Sigma); and 2.2 g sodium bicarbonate. The pH of the solution was adjusted with 1 N NaOH to 7.6; 200 ml of warm 7% gelatin (Difco) was then added. The medium was sterilized by filtration (0.2 µm nitrocellulose, Millipore Corp., Bedford, MA) with air pressure. Sterile rabbit serum ("partially hemolyzed"; Pel-Freez Biologicals, Inc., Rogers, AR) was added to a final concentration of 6%. The BSK medium was dispensed in 9 ml volumes to plastic tissue culture tubes (Falcon Division of Becton-Dickinson and Co., Oxnard, CA, no. 3033). After inoculation, the tightly capped tubes were incubated at 35°C. Cells were enumerated by the method of Stoenner [13].

The IDS originally isolated on Shelter Island [3] was cloned by limiting dilution [13,14] in fortified Kelly's medium [14] after passage of a culture sequentially through 0.45 and 0.2 µm nitrocellulose filters (Nalge Co., Rochester, NY) under vacuum. One of the cloned populations was designated strain B31 and was used to assess the BSK medium described above and as a reference strain with which to compare other ixodid tick-borne spirochetes.

* To whom reprint requests should be addressed.

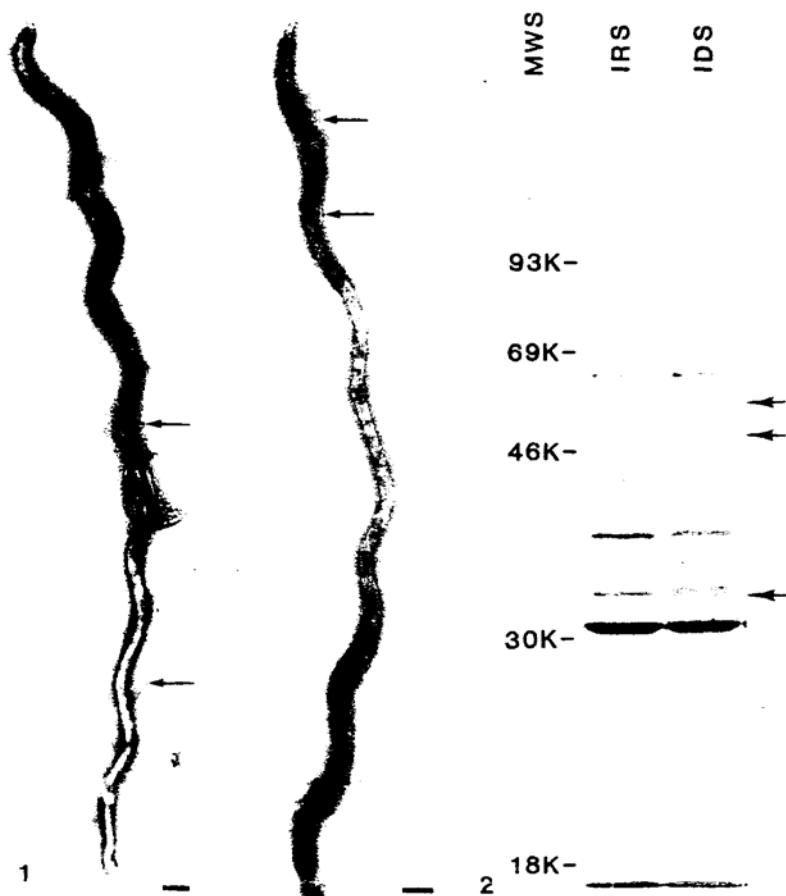


Fig. 1. Electron photomicrograph of *in vitro*-cultivated *Ixodes ricinus* spirochete (left) and *Ixodes dammini* spirochete (right). Spirochetes were negatively stained with ammonium molybdate. Arrows indicate axial filaments. Bar, 0.5 μ m.

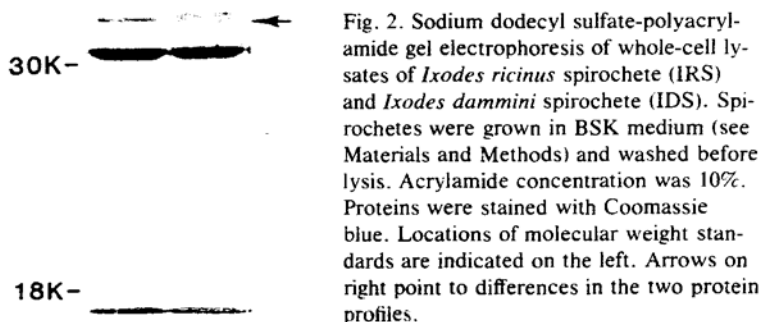


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell lysates of *Ixodes ricinus* spirochete (IRS) and *Ixodes dammini* spirochete (IDS). Spirochetes were grown in BSK medium (see Materials and Methods) and washed before lysis. Acrylamide concentration was 10%. Proteins were stained with Coomassie blue. Locations of molecular weight standards are indicated on the left. Arrows on right point to differences in the two protein profiles.

Adult *I. ricinus* ticks were collected by flagging vegetation in the Seewald forest in the canton of Bern. Details of the collection, examination, and surveys of *I. ricinus* ticks are described elsewhere [4]. The midguts from 4 *I. ricinus* ticks that were found to have spirochetes by direct examination were removed and crushed in 1 ml of BSK medium in a glass tissue homogenizer. Three-tenths ml of the suspension was inoculated into each of 3 tubes (1:30 dilution). Further dilutions (1:300, 1:3,000, and 1:30,000) were made from 2 of the 3 tubes.

Spirochetes in late-log phase BSK cultures were examined by electron microscopy. After washing the organisms twice with phosphate-buffered saline, pH 7.4 (PBS), we applied the spirochetes to parlodion-coated copper grids and stained them with 2% ammonium molybdate, pH 6.5. The grids were examined with a Hitachi 11-EU-1 electron microscope. Photographs were made on Kodak SO-163 film.

Serum from a Connecticut resident with Lyme disease was kindly provided by Dr. Allen Steere, Yale University, New Haven, CT. Convalescent serum from a patient with ECM was obtained from the Zoological Institute at Neuchâtel. Antiserum to strain B31 was raised in a New Zealand white rabbit by intravenous injections of live, washed spirochetes on days 1, 8, 14, 20, and 44. The rabbit was bled on day 52. Indirect immunofluorescence assays (IFA) for antibodies to methanol-fixed, BSK medium-grown spirochetes and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) of spirochete lysates were performed as described previously [2]. The fluorescinated goat anti-human globulin serum (BBL Division of Becton-Dick-

inson and Co., Cockeysville, MD) and goat anti-rabbit IgG serum (Miles-Yeda, Ltd., Rehovot, Israel) were used in 1:200 dilutions in 1% BSA in PBS.

Results

IDS strain B31 grew at a rate of approximately one doubling per 12 h in BSK medium at 35°C. Cultures could be started with as few as 1–2 organisms. The density of spirochetes in an early stationary phase culture was usually between 0.5–1 $\times 10^8$ per ml. The successful growth of the IDS in BSK medium encouraged us to use it for isolations of spirochetes from *I. ricinus*.

We serially examined the cultures of the *I. ricinus* midgut suspension by phase microscopy. The 1:30 and 1:300 dilution tubes contained, in addition to spirochetes, unclassified, motile, gram-positive bacilli. In contrast, the 1:3,000 and 1:30,000 dilution tubes contained only spirochetes. The presence of the spirochetes in these higher dilutions was detectable as early as 7 days after inoculation. The phase microscopic appearance of live *I. ricinus*

spirochetes (IRS) was identical to that of the IDS. Both isolates had irregular coils and relatively slow rotating movements with occasional bending. In the first few passages, the IRS were found in large clumps that quickly reaggregated and settled to the bottom of the tube after disruption. We did not see the aggregation phenomenon after 5–7 subpassages.

Examples of the IRS and IDS in negatively stained preparations for electron microscopy are shown in Fig. 1. Both strains had been passaged at least 10 times in BSK medium before examination. The IDS and IRS could not be distinguished in these preparations. The lengths ranged between 10–20 μ , and the widths were 0.18–0.25 μ .

Fig. 2 shows that the PAGE-Coomassie blue-protein profiles in whole lysates of the IRS and IDS are very similar to one another. With the exceptions of 3 minor proteins, which are identified in the figure, the PAGE profiles of the 2 isolates were identical.

Immunofluorescence studies demonstrated that the 2 isolates share common antigenic determinants as well. The following IFA reciprocal serum titers against IDS and IRS, respectively, were obtained: Connecticut Lyme disease patient, 1280 and 1280; Swiss ECM patient, 640 and 640; and rabbit immunized with B31, 2560 and 2560. Normal human sera and pre-immune rabbit sera in the same assays gave titers no greater than 40 against either organism.

Discussion

A spirochete of *I. ricinus* ticks was isolated in BSK medium. This medium shares constituents with both plain [7] and fortified Kelly's medium [14]. Like fortified Kelly's medium, it contains CMRL 1066 tissue culture medium as a source of amino acids, vitamins, and other factors that may aid cultivation of these spirochetes. In comparison with fortified Kelly's medium, BSK medium has Neopeptone as the peptone preparation, HEPES as a buffer, and no Yeastolate. The easily prepared BSK medium is also useful for growing *Borrelia hermsii* and *turicatae*, two other tick-borne spirochetes [unpublished observations, A. G. Barbour].

The IRS we have isolated appears to be closely related by criteria of morphology, PAGE protein profiles, and antigenic determinants to a spirochete of *I. dammini* ticks of northeastern United States. If United States physicians are as sensitive as their European counterparts to the appearance of new

disorders, then the relatively recent recognition of ECM and Lyme disease in the United States [8,12], after it had long been noted in Europe [1,5], suggests that the etiologic agent of this disorder may have been introduced to North America from across the Atlantic. The marked similarity between the IDS, which appears to be the etiologic agent of Lyme disease [3], and the IRS, which was isolated from ticks in an area where ECM is endemic, also indicates that such an introduction may have been, on an evolutionary time scale, very recent. Further studies on the lineage of the ixodid tick-borne spirochetes are planned.

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