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Isolation of *Borrelia burgdorferi* from Saliva of the Tick Vector, *Ixodes scapularis*†

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A method for cultivating and isolating Lyme disease spirochetes, *Borrelia burgdorferi*, from the saliva of vector ticks, *Ixodes scapularis* (formerly known as *Ixodes dammini*), is described. Saliva was collected from partially engorged ticks after application of pilocarpine to induce salivation. *B. burgdorferi* was isolated from 8 of 14 (57%) of the saliva samples derived from ticks infected with the bacteria, as determined by direct immunofluorescent-antibody assay of tick hemolymph. A comparison of the protein profiles of the salivary isolates and a highly passaged strain (B31) showed that the salivary isolates all lacked a 22-kDa protein known to increase with continuous passage, but exhibited larger amounts of the OspA and OspB proteins than did the highly passaged B31 strain.

Lyme disease is caused by a systemic spirochetal infection transmitted by ixodid ticks while they feed on blood (9). Shortly after the onset of blood feeding, Lyme disease spirochetes (*Borrelia burgdorferi*) migrate through the tick gut epithelium and pass into the hemolymph and then on to various tissues including the salivary glands (6, 13, 23, 28). Spirochetes have been visualized in tick saliva by fluorescent-antibody assay (23). While such strong evidence largely supports a salivary route of transmission for *B. burgdorferi*, this point has been debated. Alternatively, it has been suggested that spirochetes may be introduced into the host by regurgitation from the midgut (10). Spirochetes disseminating from the tick midgut likely encounter a variety of biochemical, enzymatic, and cellular conditions, some of which may be hostile to the spirochete (10). Disseminated spirochetes may not survive, or they may survive only transiently in vector tissues and organs. Dissemination through the tick may also change the spirochete antigenically. Thus, the mode by which spirochetes transfer to hosts may affect host infection as well as the host immune response (12).

Spirochetes have been cultivated from many different tissues of reservoir animals (2, 25, 26), animal models (3, 19), and humans (7, 27) as well as by macerating ticks (2, 25). While the efficiency with which new isolates are obtained from various tissues has improved greatly since the earliest attempts at cultivating Lyme disease spirochetes, culture contamination and slow growth of the primary culture continue to be problems.

Tick saliva contains a mixture of pharmacologically active components. Several of these components have been identified, characterized, and shown to enhance the tick blood-feeding process (20, 22). Additionally, *Ixodes scapularis* (formerly *Ixodes dammini*) tick saliva may inhibit the host immune response, thereby promoting infection by *B. burgdorferi* (21). In this report, we describe the cultivation of *B. burgdorferi* from the saliva of partially or freshly engorged *I. scapularis* ticks. We also examine the protein profiles of several of the salivary isolates, comparing them with each other and with a strain (B31) cultivated for several years in laboratory medium.

MATERIALS AND METHODS

Adult *I. scapularis* ticks were collected by flagging at three locations during the fall of 1992. Sampling sites were located in Rhode Island (Webster, Charlestown), Massachusetts (Ipswich), and Pennsylvania (Bryn Athyn). High rates of spirochete prevalence have previously been reported for each sampling location (1, 16, 18). All ticks were stored in vials and were maintained at 98% humidity and 5°C for up to 5 months before inducing salivation.

To prepare the ticks to salivate, they were allowed to engorge partially by feeding on the blood of one of two New Zealand White rabbits. In the first trial, 40 mating pairs of ticks from Ipswich were placed on one ear and 40 mating pairs from Webster (Charlestown) were placed on the other ear. In the second trial, 40 mating pairs from Pennsylvania were allowed to feed on a single ear. Ticks were contained on the rabbit's ear by using cloth bags affixed with tape. Mostly replete *I. dammini* ticks were harvested from the first rabbit on the fifth, sixth, and seventh days postattachment. All ticks were harvested from the second rabbit on the sixth day postattachment.

Upon harvesting, ticks were rinsed in distilled water and were then immediately fixed to glass slides with double-sided tape, and a sterile glass micropipette was placed around the hypostome to collect saliva (Fig. 1). Salivation was induced by the application of 2 μl of pilocarpine (50 mg/ml in 95% ethanol) to the scutum of the tick (23). Additional 1-μl aliquots of pilocarpine were applied at 20-min intervals when little salivation was observed. Ticks were incubated at 35°C in a humid chamber until salivation ceased (2 to 3 h). Micropipettes were removed from the ticks, and the amount of saliva collected was determined. Typically, volumes ranged from 10 to 20 μl per tick. Saliva was then inoculated directly into 1.5-ml screw-cap tubes containing 1 ml of BSKII medium (4) plus either rifampin (50 μg/ml) or kanamycin (8 μg/ml).

After collecting saliva, a hemolymph sample was obtained from each tick by clipping a front leg and letting the tick "bleed" onto a clean glass slide. Hemolymph samples were air-dried, fixed for 10 min in acetone, and then examined at ×400 magnification for the presence of *B. burgdorferi* by using

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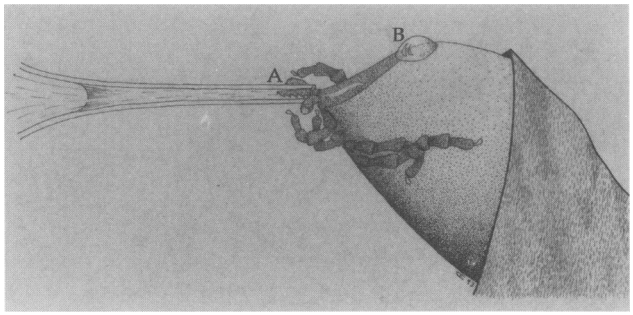

FIG. 1. Method used to collect saliva from ticks. (A) Tick hypostome inserted into sterile glass micropipette. (B) Pilocarpine applied to scutum of tick.

a direct fluorescent-antibody assay (17). The tubes of media containing tick saliva were incubated at 33°C and were examined periodically by phase-contrast microscopy for the presence of spirochetes. When growth was noted, cells were passed every 5 days in 12 ml of BSKII medium without antibiotics. After 5 to 6 weeks of passage, frozen stocks were made by adding 0.15 ml of 90% glycerol to 0.85 ml of cell culture and storing at −70°C. For later comparisons, *B. burgdorferi* B31 was cultured in BSKII medium without antibiotics.

Protein profiles of whole-cell lysates from salivary isolates I2 (Ipswich tick 2), I3, and W7 (Webster, Charlestown, tick 7) were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15) and were compared with the high-passage strain, strain B31. Cells were grown in 5 ml of BSKII medium and were washed in RPMI (GIBCO, Gaithersburg, Md.). The washed cell pellets were resuspended in 100 μl of RPMI and were broken by 10 s of continuous sonication (setting 5 on Sonifier Cell Disruptor model 350; Branson Sonic Power Co.). Protein concentrations were measured by the assay of Bradford (8). Equal amounts of protein (8 μg) from each sample were solubilized in sample buffer (11, 15). The proteins were then separated by SDS-PAGE on a 12% polyacrylamide gel (15).

The protein profiles of two salivary isolates, I4 and W2, labeled with [^{35}S]methionine were examined by SDS-PAGE and fluorography and were compared with that of *B. burgdorferi* B31. Cells grown in BSKII medium (30 ml) at 33°C were washed three times in RPMI without methionine and were resuspended in 400 μl of RPMI without methionine (11). The cells were then labeled with 10 μCi of [^{35}S]methionine (specific activity, 800 Ci/mmol) for 1 h at 33°C (11).

RESULTS AND DISCUSSION

Active spirochetes were observed by phase-contrast microscopy as early as 3 days after inoculating BSKII medium with tick saliva (Table 1). At 6 days, the first visible cell pellet appeared in the culture tube containing saliva from tick I4, at which time the cells were subcultured. The last sample to be subcultured (I18) was at 25 days after inoculation. In all cases in which saliva-inoculated medium produced *B. burgdorferi*, the cells grew in large clumps at the bottom of the culture tubes. However, after subculturing for 5 to 6 weeks, *B. burgdorferi* cells were observed growing at a high density throughout the culture, similar to the growth of highly passaged strain B31. Prior to reaching this stage of dispersed growth, the isolates from tick saliva were unable to survive freezing, even in the presence of glycerol. Phase-contrast microscopic observation of the salivary isolates suggested that they exhibited a higher rotation rate than the high-passage strain, strain B31.

TABLE 1. Time required to cultivate *B. burgdorferi* isolates from tick saliva

Tick and isolate no.[a]	No. of days after:		Spirochetes detected in tick hemolymph
	Tick attachment that saliva was collected	Inoculated spirochetes were detected in culture	
I4	5	3	+
P1	6	6	+
I2	5	8	+
W2	5	8	+
I11	6	14	+
P2	6	20	−
I3	5	21	+
W7	6	21	+
I18	6	25	+

[a] I, Ipswich, Mass.; P, Bryn Athyn, Pa., W, Webster, Charlestown, R.I.; numbers are isolate number for each location.

We were able to identify *B. burgdorferi* in hemolymph from 14 of the 42 ticks tested. Of these 14 ticks, 8 (57%) gave cultivable spirochetes from their saliva. In one case, *B. burgdorferi* was cultured from tick saliva but no spirochetes were detected in the hemolymph (Table 1). Our ability to detect spirochetes in tick hemolymph is likely lower than our ability to detect spirochetes in tick midguts, but a previous study (23) indicated that spirochetes can be detected only in the saliva of hemolymph-positive ticks.

Coomassie blue and silver stainings of whole-cell protein lysates separated by SDS-PAGE showed lower amounts of proteins OspA and OspB in strain B31 compared with the amounts in the salivary isolates (Fig. 2). Previous reports indicate that OspB is lost during passage of *B. burgdorferi* (24). Isolate W7 had two proteins with molecular masses of approximately 50 and 44 kDa that were not present in the other isolates examined or in strain B31. Isolates I2 and I3 each had a protein band at 30 kDa that was not present in isolate B31 or isolate W7. Conversely, a 22-kDa protein was detected in isolate B31 but was not found in any of the salivary isolates. This result is consistent with that of previous work (24), which demonstrated a relative increase in a lower-molecular-weight protein with continued laboratory culture of *B. burgdorferi*. These were the only differences observed in the stained gels.

Examination of the [^{35}S]methionine-labeled protein profiles from isolates I4, W2, and B31 showed few differences except in the low-molecular-weight region (Fig. 3). OspA and a protein of about 25 kDa were present in reduced amounts or were absent from B31. Additionally, isolates I4 and W2 each exhibited a large band at approximately 8 kDa which was not detected in B31. It is interesting that this band was not apparent in any strain in Coomassie blue- or silver-stained gels.

Here we reported a novel method of cultivating *B. burgdorferi* directly from tick saliva. The cultivation time and the difficulties with contamination were substantially decreased (25). Success in cultivating spirochetes directly from saliva also further supports the hypothesis that *B. burgdorferi* is transmitted via the salivary glands, as opposed to via regurgitation from the midgut. Decreased levels of contamination (just 7 contaminated cultures of 42 culture attempts) and a shortened cultivation time as a result of culturing spirochetes directly from the saliva may increase the efficiency with which primary isolates can be obtained for research efforts such as strain typing and other epizootiologic studies (25).

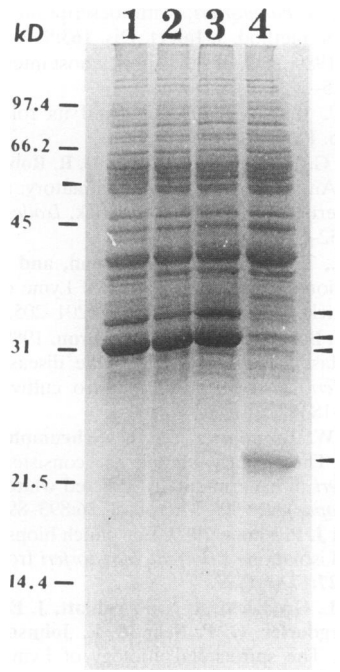

FIG. 2. Comparison of proteins from three tick salivary isolates of *B. burgdorferi* and strain B31. Equal amounts of protein from each strain (lane 1, W7; lane 2, I3; lane 3, I2; lane 4, B31) were loaded onto each lane and were separated by SDS-PAGE. Arrowheads indicate the specific proteins that differed between strains. Molecular size standards are in kilodaltons.

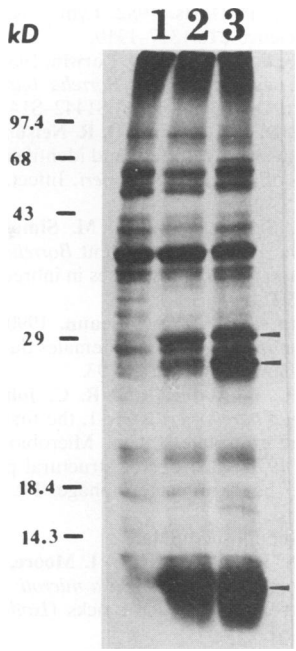

FIG. 3. Fluorogram of [^{35}S]methionine-labeled proteins from two salivary isolates of *B. burgdorferi* and strain B31. Equal counts per minute from each strain (lane 1, B31; lane 2, I4; lane 3, W2) were loaded onto each lane and were separated by SDS-PAGE. Arrowheads indicate the specific proteins that differed between strains. Molecular size standards are in kilodaltons.

B. burgdorferi isolates destined to infect the host via the salivary route disseminate from the tick midgut to the hemocoele during feeding and eventually appear in the saliva. Tick saliva has many pharmacological components that probably aid the infectivities of the spirochetes. These include chemicals with anticomplement, anti-inflammatory, and antihistaminic activities as well as a variety of other factors that inhibit the host immune response (20–22). Delivery of *B. burgdorferi* by tick saliva into the skin of a susceptible host may enhance establishment of the spirochete in tissues surrounding the tick bite by temporarily inhibiting host immune responses. Incubation in or passage through tick saliva may also affect the antigenic profile of *B. burgdorferi*. In addition to transporting the spirochetes during transmission from tick to host, saliva is probably also present intradermally. Whether tick saliva has any effect on the physiology of Lyme disease spirochetes, either in the tick or at the bite site, is under investigation.

The phenomenon of the clumping together of *B. burgdorferi* isolates is characteristic of newly cultured isolates (5). The motilities of the isolated spirochetes appear to increase with passage in laboratory medium, and motility is accompanied by a decrease in the amount of clumping (5). It would be interesting to know whether this phenomenon in vitro can be correlated with the process of dissemination in the skin during the early stages of the disease.

Differences in the protein and DNA profiles of *B. burgdorferi* isolates have been reported by several investigators, and variations in the protein and DNA profiles among isolates also occur (14, 24). However, the most notable differences between the salivary isolates of *B. burgdorferi* and high-passage strain B31 are those commonly noted between low- and high-passaged isolates and include the relative lack of OspA and OspB proteins in isolate B31 and the absence of a 22-kDa protein in the salivary isolates.

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