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Lyme Disease: a Selective Medium for Isolation of the Suspected Etiological Agent, a Spirochete

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A simple procedure with a new selective culture medium for the isolation of the suspected etiological agent of Lyme disease from ticks is described. Live ticks (*Ixodes dammini*) were ground with a mortar and pestle, and the suspensions were inoculated into a selective and nonselective medium. The selective medium, which contained kanamycin and 5-fluorouracil, yielded positive spirochete cultures from 100% of the pooled ticks and from 79% of the single tick specimens. The isolation rate for the nonselective medium was 0% from the tick pools and 58% from the single tick specimens.

Lyme disease is a systemic illness characterized by a distinctive skin lesion (erythema chronicum migrans) often followed by neurological, cardiac, or arthritic complications or any combination of the three (7, 8, 10). A spirochete, thought to be the etiological agent of Lyme disease, has been recovered from ticks (*Ixodes dammini* and *Ixodes ricinus*) (1, 4) and from patients with Lyme disease (3, 9). The spirochete was recovered from ticks by inoculation of the isolated midgut into a modified Kelly medium (4). The problems with this procedure are that dissection of the tick to remove the midgut is a tedious and time-consuming procedure which limits the number of ticks that can be tested, and overgrowth by contaminating bacteria often occurs. Our solutions to these problems were to shorten the time required for the preparation of an inoculum by grinding the whole tick with a mortar and pestle and to use a selective medium to prevent overgrowth of bacterial contaminants.

Culture media. Various modifications of Kelly medium (5) have been used to grow the Lyme disease spirochete. We used the BSK medium described by Barbour et al. (1). (BSK is a term used by the authors who described the medium. In their original paper describing this medium, BSK is the only term used. BSK medium is a modification of "fortified Kelly medium" described by Stoenner et al. [11].) BSK medium was prepared with the following: 900 ml of double-distilled water, 100 ml of 10× Connaught Medical Research Laboratories 1066 liquid medium with glutamine (6) (no. 330-1545; GIBCO Laboratories, Grand Island, N.Y.), 5 g of neopeptone (Difco Laboratories, Detroit, Mich.), 50 g of bovine serum albumin fraction V (no. 81-003; Miles Laboratories, Elkhart, Ind.), 6 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid buffer (Sigma Chemical Co., St. Louis, Mo.), 0.7 g of sodium citrate, 5 g of glucose, 0.8 g of sodium pyruvate (Sigma), 0.4 g of *N*-acetylglucosamine (Sigma), and 2.2 g of sodium bicarbonate. Each ingredient was dissolved as added, then the solution was adjusted to pH 7.6 with 1 N NaOH. A 200-ml volume of warm 7% aqueous gelatin (Difco) solution was added, and the completed medium was sterilized by filtration through a membrane filter. Sterile rabbit serum ("partially hemolyzed"; Pelfreez Biologicals, Inc., Rogers, Ariz.) was added to final serum concentration of 6%. The selective medium BSK-K5, was prepared by adding kanamycin (Bristol Laboratories, Syracuse, N.Y.) and 5-fluorouracil (Roche Laboratories, Nutley, N.J.) to the BSK medium in a final concentration of 8 and 230 μg/ml, respectively. The final pH of the medium was 7.7 after all ingredients were added. The selective and nonselective media were prepared from the same batch of BSK medium and dispensed into plastic, screw-cap tissue culture tubes (13 by 100 mm; Falcon no. 2027; Oxnard, Calif.) in a volume of 6.6 ml per tube leaving an air space of 16 mm above the top of the medium. The amount of air space in the tube appears to have an effect on growth of the spirochete.

Medium sensitivity: BSK-K5 versus BSK. We compared BSK-K5 medium with BSK medium and found that both media supported growth of approximately one Lyme disease spirochete, strain B31 (1), which was detectable within 10 days after inoculation. We also found that spirochete growth was detectable in both media from approximately 13 spirochetes within 7 days and 130 spirochetes within 4 days after inoculation. All initial detectable growth was observed within 10 days of inoculation, even though all cultures were examined periodically for 21 days.

Tick suspensions. *I. dammini* was collected from lower vegetation in Monmouth County, N.J., during October and November 1982. Ticks were placed in 1-dram (ca. 60-g) screw-cap vials, one tick per vial, with approximately 0.5 ml of 70% isopropanol. The vial was swirled for 3 or 4 s, and the ticks were removed and dried on sterile gauze squares. Live ticks were placed in a small sterile mortar containing 0.5 ml of BSK broth and ground with a pestle. Additional BSK medium was added to increase the volume to 2.0 ml for the pooled tick specimens and to 1.0 ml for the single tick specimens. A total of 7 suspensions was prepared from pooled ticks (five ticks per pool), and 19 suspensions were prepared from single ticks.

Cultivation procedure. A pooled tick suspension (0.2 ml) or 0.1 ml of a single tick suspension was inoculated into both BSK-K5 and BSK medium. A stock culture of the Lyme disease spirochete, strain B31, was inoculated into both media as controls. The caps were tightened to prevent air exchange, and the cultures were incubated at 33°C. Samples from the culture tubes were examined between 3 and 13 days for motile spirochetes by dark-field microscopy.

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TABLE 1. Recovery rate of the Lyme disease spirochete from pooled and single tick specimens

Tick specimens	No.	Media			
		Selective (BSK-K5)		Nonselective (BSK)	
		No. positive (%)	No. contaminated (%)	No. positive (%)	No. contaminated (%)
Pooled (5/pool)	7	7 (100)[a]	3 (42.9)	0 (0)[b]	7 (100)
Single	19	15 (78.9)[c]	0 (0)	11 (57.9)[d]	8 (42.1)

[a] Six (85.7%) were positive within 6 days; the seventh was positive within 9 days.
[b] None were positive within 13 days.
[c] Ten (52.6%) were positive within 6 days; the remaining five were positive within 13 days.
[d] Six (31.6%) were positive within 6 days; the remaining five were positive within 13 days.

We chose not to examine the cultures after 13 days based on our results from the medium sensitivity study.

All isolates strongly reacted (4+) to a monoclonal fluorescent antibody specific for the Lyme disease spirochete, strain B31, kindly supplied by Alan G. Barbour (2), Rocky Mountain Laboratories, Hamilton, Mont. All isolates were successfully subcultured to BSK medium for maintenance of the individual strains in the stock collection.

Growth of the Lyme disease spirochete was detected in BSK-K5 medium within 3 to 6 days with six (85.7%) of the seven pooled tick specimens and within 9 days for the remaining specimen, resulting in a recovery rate of 100% (Table 1). No growth of the Lyme disease spirochete was detected in the BSK medium; however, growth of bacterial contaminants did occur in all of the tubes of BSK medium within 3 to 6 days after inoculation. The early appearance of bacterial growth may have inhibited the Lyme disease spirochete in the BSK medium. Stock cultures of the Lyme disease spirochete, which were inoculated into BSK and BSK-K5 media as controls, grew equally well in both media within 3 days.

In a second experiment, the Lyme disease spirochete was isolated within 3 to 6 days from 10 of the 19 single tick specimens in BSK-K5 medium and from 6 of the 19 single tick specimens in BSK medium. The spirochete was isolated from five additional specimens within 9 to 13 days in both the BSK and BSK-K5 media. Therefore, the recovery rate of the spirochete from single tick specimens was 79% in the BSK-K5 medium and 58% in the BSK medium. This difference was not significant ($P = 0.148$, Fishers exact test); however, the difference in the recovery rate of the spirochete from BSK-K5 medium compared with the BSK medium was significant when the pooled tick results were combined with the single tick results ($P = 0.00171$, Fishers exact test). In no instance was the spirochete recovered from BSK medium but not from BSK-K5 medium. The pH of the media was 7.0 to 7.7 after spirochete recovery, as indicated by the phenol red indicator present in the Connaught Medical Research Laboratories 1066 component of the medium.

Although the rate of bacterial contamination was less with the single tick specimens compared with the pooled tick specimens, it was still a problem. Growth of bacterial contaminants occurred within 6 days in the BSK medium with 42% of the single tick specimens, whereas 0% were contaminated in BSK-K5 medium.

Our findings indicate that (i) grinding *I. dammini* ticks with a mortar and pestle is a suitable method for the preparation of suspensions for the isolation of the Lyme disease spirochete, and (ii) the BSK-K5 medium is superior to the BSK medium for the isolation of the Lyme disease spirochete from pooled or single tick specimens because it prevents overgrowth by bacterial contaminants, yet it supports the growth of the Lyme disease spirochete as well as the BSK medium.

The selective medium, BSK-K5, was developed for isolating the Lyme disease spirochete from contaminated material such as ticks. However, the use of this medium could be extended into the clinical laboratory for isolating the spirochete from patient material (i.e., skin biopsies, blood, and cerebral spinal fluid) which have accidentally become contaminated.

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LITERATURE CITED

1. **Barbour, A. G., W. Burgdorfer, S. F. Hayes, O. Peter, and A. Aeschlimann.** 1983. Isolation of a cultivatible spirochete from *Ixodes ricinus* ticks of Switzerland. Curr. Microbiol. **8**:123–126.
2. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41**:795–804.
3. **Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, L. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow.** 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. **308**:740–742.
4. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216**:1317–1319.
5. **Kelly, R.** 1971. Cultivation of *Borrelia hermsii*. Science **173**:443–444.
6. **Parker, R. C., L. N. Castor, and E. A. McCulloch.** 1957. Altered cell strains in continuous culture: a general survey, p. 310. *In* Cellular biology and nucleic acids and viruses. Special publications of the New York Academy of Sciences, vol. V. New York Academy of Sciences, New York.
7. **Reik, L., A. C. Steere, N. H. Bartenhagen, R. E. Shope, and S. E. Malawista.** 1979. Neurologic abnormalities of Lyme disease. Medicine **58**:281–294.
8. **Steere, A. C., W. P. Batsford, M. Weenbert, J. Alexander, H. J. Berger, S. Wolfson, and S. E. Malawista.** 1980. Lyme carditis: cardiac abnormalities of Lyme disease. Ann. Intern. Med. **93**:8–16.
9. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308**:733–740.
10. **Steere, A. C., S. E. Malawista, J. A. Hardin, S. Ruddy, P. W. Askenase, and W. A. Andiman.** 1977. Erythema chronicum migrans and Lyme arthritis: the enlarging clinical spectrum. Ann. Intern. Med. **86**:685–698.
11. **Stoenner, H. G., C. Larson, and T. Dodd.** 1982. Antigenic variation of *Borrelia hermsii*. J. Exp. Med. **156**:1297–1311.