JOURNAL OF CLINICAL MICROBIOLOGY, May 1993, p. 1251–1255
0095-1137/93/051251-05$02.00/0

Vol. 31, No. 5

Standardization of Medium for Culturing Lyme Disease Spirochetes

R. J. POLLACK,* S. R. TELFORD III, AND A. SPIELMAN

Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts 02115

Received 16 December 1992/Accepted 17 February 1993

To standardize the procedure for isolating and culturing Lyme disease spirochetes, we modified the composition of the medium generally used for this purpose (BSK-II) and developed a system for its distribution. This medium contains no gelatin or agarose, and various components are used in proportions that differ from those in BSK-II. Each of the major proteinacious components was screened by substitution in samples of the complete product. The final medium was evaluated for the capacity to grow related spirochetes including *Borrelia burgdorferi* N40, Guilford, and JD-1 as well as strains of *Borrelia hermsii* (HS-1) and of *Borrelia coriaceae* (CO53). Each isolate developed from inocula containing as few as one to five organisms. Doubling time of *B. burgdorferi* during log-phase growth at 37°C was 10 to 12 h. Lyme disease spirochetes were isolated in this medium from ear punch biopsies and dermal aspirates from naturally infected mice and rabbits, from dermal biopsies from a human patient, and by sampling field-collected deer ticks (*Ixodes dammini*). Cultured spirochetes remained infective to mice and to ticks. The medium can be stored at −20°C or lower temperatures for at least 8 months without effect on its ability to support growth of small inocula to densities exceeding 10^8 spirochetes per ml. Lyme disease spirochetes remained infective to mice after being stored at −80°C in this medium for at least 8 months. We anticipate that the availability of this standardized medium (Sigma Chemical Co.), supplemented with prescreened rabbit serum, will facilitate comparison of research results between laboratories and may eventually permit definitive clinical diagnosis of Lyme disease based on demonstration of the pathogen. The standardized medium is designated BSK-H.

Efforts to culture the Lyme disease spirochete, *Borrelia burgdorferi*, generally depend upon the use of certain complex media, mainly BSK-I and BSK-II (named for Barbour, Stoenner, and Kelly) (2). Such media have facilitated production of spirochete antigen and generally serve as a basis for detecting these pathogens in clinical specimens (10) as well as in vector and reservoir hosts (1). Indeed, the seminal demonstration of the spirochetal etiology of Lyme disease used antiserum raised against vector-derived spirochetes that were cultured in such a medium (3). To be useful for definitive clinical diagnosis, a medium should be stable, readily available, and uniformly efficacious and must satisfy regulatory requirements.

Various problems, however, diminish the usefulness of existing media. No commercial source distributes these formulations. Their preparation requires incorporation of such separate and highly variable components as bovine serum albumin (BSA), neopeptone, yeast extract, and rabbit serum into the commercial tissue culture medium CMRL-1066 that serves as the base (2). Indeed, batch variation in media prepared by using different sources of BSA influenced growth kinetics, morphology, and antigenic characteristics of Lyme disease spirochetes (4). The quality of these proteinacious, densely particulate components is so variable that each requires prescreening and special care in preparation and filtration. Batches of prepared media frequently must be discarded, because they do not support the growth of borreliae. Under these constraints, only specialized research laboratories can reliably isolate Lyme disease spirochetes or produce them for experimental or diagnostic purposes.

* Corresponding author.

We reasoned that the ready availability of a standard medium that effectively supports the growth of Lyme disease spirochetes would facilitate work on these and related pathogens. Toward this end, we sought to develop a standardized medium for the isolation and maintenance of the agent of Lyme disease as well as other borreliae and to take steps to promote its availability. Such a medium should permit growth from an inoculum of as few as one to five organisms and permit the inoculum to double in 10 to 12 h and to reach densities of at least 10^8 organisms per ml (2). This study was designed to provide a standardized medium suitable for experimental use; observations of a single human patient were included in anticipation of a future clinical trial.

MATERIALS AND METHODS

Spirochetes. The N40 strain of *B. burgdorferi* that was used throughout this study was maintained by serial passage in BSK-II medium. Unless otherwise indicated, the term "Lyme disease spirochete" refers to this strain. A division of this strain was maintained in a natural cycle of transmission between its natural deer tick vector, *Ixodes dammini*, and its white-footed mouse reservoir host, *Peromyscus leucopus*. Two other strains of Lyme disease spirochetes that were used in one experiment (Table 1) were routinely maintained by serial passage in BSK-II. A strain of *Borrelia hermsii* (courtesy of T. Schwan, Rocky Mountain Laboratory) and a strain of *Borrelia coriaceae* (ATCC 43381) were also used in this work.

Formulation. Endotoxin-tested tissue culture grade water was used to dissolve components of experimental media. The formulation of this medium is provided in Table 2. Medium components for preliminary tests were acquired

TABLE 1. Spirochetes used to evaluate experimental media

Species	Strain	No. of passages
B. burgdorferi	N40 (culture adapted)	7–10
	N40 (tick adapted)	7–10
	Guilford	49–53
	JD-1	4–25
B. hermsii	HS-1	>20
B. coriaceae	CO53	>20

from diverse commercial sources. All components utilized in the final preparation were supplied by Sigma Chemical Co., Cell Culture division, St. Louis, Mo. The pH was adjusted to 7.6 at 25°C with 1 N NaOH; osmolality was 435 ± 25 mosmol/kg of H_2O. Media preparations were sterilized by filtration through a membrane with a porosity of less than 0.22 μm. Prior to use, media were supplemented with rabbit serum (Sigma R-7136) to a final concentration of 6% and stored at −80, −20, 4, 33, and 37°C for later analysis. Sera were stored at −20°C.

We determined whether certain lots of commercially obtained rabbit serum contain antibody that reacts with spirochetal antigens by enzyme-linked immunosorbent assay (ELISA), as previously described (11). Briefly, 96-well immunoassay plates were coated overnight with 50 μl of a soluble fraction of a whole-cell sonicate of *B. burgdorferi* JD-1 at 10 μg/ml and blocked with 5% horse serum. A preparation containing treponemal group antigens (Bacto FTA sorbent; Difco) was used at the identical concentration to coat comparison plates. Test sera were diluted 1:100 in phosphate-buffered saline (PBS)-horse serum, plated in triplicate, and incubated for 1 h at 37°C. Secondary antibody (alkaline phosphatase-labeled anti-rabbit immunoglobulin G; Sigma) was used according to the manufacturer's directions. The substrate was *p*-nitrophenyl phosphate, 5 mg/ml in diethanolamine buffer; reactions were terminated at 30 min by the addition of 3 N NaOH and promptly read at 405 nm. A positive control sample from a rabbit which had served as host to infected adult *I. dammini* 2 months earlier was included on each plate. The negative control sample was a pool of serum taken from three rabbits prior to their use as hosts for ticks. This serum did not react with spirochetal proteins by immunoblotting (data not shown). Cutoff values were derived by calculating the mean of the optical density of 12 wells containing the negative-control rabbit serum plus 3 standard deviations.

Culture conditions and evaluation. Spirochetal growth dynamics in evacuated blood collection tubes (Vacutainer 6380; Becton Dickinson, Rutherford, N.J.) containing 2 ml of medium each were compared. Triplicate cultures were incubated in the dark at 33 and at 37°C. Spirochetes were counted periodically following gentle agitation, and gross morphology and relative motility were assessed by dark-field microscopy using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, Pa.). Spirochetes were sampled aseptically by removing 5 to 10 μl of medium with a fine hypodermic needle to preserve the gas balance within the tubes. Three replicate counts of spirochetes were made for each test at each sampling period.

The growth dynamics of spirochetes were evaluated in parallel for triplicate cultures in wells of 96-well microtiter plates (Corning model 25860; Corning Glass Works, Corning, N.Y.) containing 0.2 ml of medium per well. Plates were

TABLE 2. BSK-H formulation

Ingredient[a]	mg
L-Alanine	25
L-Arginine	57.87
L-Aspartic acid	30
L-Cysteine-HCl	260
L-Cystine	20
L-Glutamic acid	75
Glycine	50
L-Histidine-HCl (monohydrate)	20
trans-4-Hydroxy-L-proline	10
L-Isoleucine	20
L-Leucine	60
L-Lysine-HCl	70
L-Methionine	15
L-Phenylalanine	25
L-Proline	40
L-Serine	25
L-Threonine	30
L-Tryptophan	10
L-Tyrosine	40
L-Valine	25
L-Ascorbic acid	50
PABA	0.05
D-Biotin	0.01
Choline chloride	0.5
Citric acid (tetrasodium)	700
Coenzyme A	2.5
Cocarboxylase	1
2′-Deoxyadenosine	10
2′-Deoxyguanosine	10
2′-Deoxycytidine-HCl	11.6
Flavin adenine dinucleotide (disodium)	0.106
Folic acid	0.01
myo-Inositol	0.05
5-Methyldeoxycytidine	0.1
β-NAD	7
β-NADP (sodium)	1
Niacinamide	0.025
Nicotinic acid	0.025
D-Pantothenic acid (hemicalcium)	0.01
Pyridoxal-HCl	0.025
Pyridoxine-HCl	0.025
Pyruvic acid (sodium)	800
Riboflavin	0.01
Thiamine-HCl	0.01
Thymidine	10
Uridine-5-triphosphate (sodium)	1
Calcium chloride (anhydrous)	200
Magnesium sulfate (anhydrous)	97.69
Potassium chloride	400
Sodium acetate (anhydrous)	50
Sodium chloride	6,800
Sodium bicarbonate	2,200
Sodium phosphate monobasic (anhydrous)	122
D-Glucose	6,000
Phenol red (sodium)	21.24
Glutathione (reduced)	10
N-Acetyl-D-glucosamine	400
D-Glucuronic acid (sodium)	3.88
Cholesterol	0.2
Polyoxyethylene sorbitan monooleate (Tween 80)	5,000
HEPES	6,000
Neopeptone	5,000
Yeast extract	2,000
BSA (fraction V)	50,000

[a] PABA, *p*-aminobenzoic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

maintained in a humidified glass desiccator jar used as a candle jar inside a 37°C incubator.

To culture spirochetes, ticks were individually washed in 70% ethanol (10 min) and sterile distilled water (three washes, 60 s each) and then placed in 50 μl of sterile PBS or in the experimental medium. Ticks were crushed by using a sterile pestle, and 25 μl of the resulting material was used to inoculate cultures. We detected spirochetal infection in ticks by use of a direct fluorescent-antibody procedure (6). Thus, 5 μl of tissue fluid was transferred to a microscope slide, and the preparation was dried, fixed in acetone, stained with fluorescein isothiocyanate-conjugated rabbit antiborrelia serum (polyclonal for defined strains of borreliae and monoclonal H5332 for field-collected spirochetes), washed in PBS, coverslipped, and examined.

Spirochetes were also cultured from biopsy specimens of mammalian skin. Samples of skin from the ears of mice, previously fed upon by infected ticks, were obtained by means of 2- and 4-mm biopsy punches. The full depth of the pinna was sampled, and the plug of tissue was washed in 70% ethanol and rinsed in PBS before transfer to a well of a microtiter plate containing the experimental medium. Plates were incubated as described above. Skin from the backs of infected mice and that from the backs of infected rabbits were similarly sampled. All animals were anesthetized; mice received 0.2 to 0.3 ml of a 0.1% pentobarbital solution intraperitoneally, and rabbits received 35 mg of ketamine-HCl and 1 mg of xylazine-HCl per kg of body weight intramuscularly. Studies were in accord with the guidelines of the Harvard Medical Area Standing Committee on Animals. A sample of skin was taken from the advancing edge of an erythema migrans lesion of a resident of Massachusetts, who had acquired infection locally but had not yet received antibiotic therapy. The site was anesthetized locally with 1% lidocane and aseptically sampled. When antibiotics were required to supplement the medium, rifampin (50 μg/ml), amphotericin B (2.5 μg/ml), and phosphomycin (20 μg/ml) were used (9).

RESULTS

Development of the formulation. In a series of preliminary studies, the original composition of BSK-II medium was modified in order to simplify its preparation. These changes included deletion of gelatin and the use of various ingredients in proportions that differed from those in the original formulation. In a preliminary study, the efficacy of the modified medium was tested by comparing the growth kinetics of an inoculum of the Lyme disease spirochete with Barbour's (2) criteria for a satisfactory medium. Indeed, one to five spirochetes became established, multiplied with a doubling time of less than 12 h during log-phase growth, and attained a density of at least 10^8 organisms per ml within 14 days of incubation. We concluded that the simplified formulation met the criteria for an effective medium for isolating and propagating the agent of Lyme disease.

We then developed a procedure for screening the components of experimental media based on the growth-supporting characteristics of the finished product. Thus, supplies of medium components were placed in reserve in bulk and tested after they were combined into modified complete media. More than 30 batches of these experimental preparations were evaluated. Media differed mainly in the sources of BSA and of the rabbit serum supplement but also in the addition, deletion, or change in concentration of various other components of the BSK-II formulation. In a preliminary evaluation of these modified media, 5×10^5 spirochetes of the culture-adapted N40 strain were inoculated into culture tubes. Spirochetes grew within 14 days in fewer than 25% of the tested preparations. The formulation that best preserved motility (frequent and active rotation with translation) and gross morphology (straight coiling, relatively uniform length, and minimal aggregation) and permitted spirochetes to develop most rapidly and abundantly was then designated as the experimental medium and was evaluated in detail.

Serum antibody. We assayed several lots of commercially obtained rabbit sera by ELISA for antibody reactive against spirochetal antigens. Of six lots of sera, one reacted with whole-cell JD-1 sonicate (optical density, 0.470; cutoff, 0.383). This sample also reacted intensely with treponemal group antigens (optical density, 0.520; cutoff, 0.338). The complete medium supplemented with this serum failed to sustain borrelial growth.

Growth kinetics. We measured the sensitivity and growth dynamics of the experimental medium in terms of the multiplication of a minimum inoculum of N40 spirochetes already adapted to BSK-II medium. Nominal inoculum densities were 0, 1, 2, 5, 10, 10^2, 10^3, and 10^4 spirochetes per culture. Culture tubes of media were inoculated in triplicate for each inoculum size, and the complete series was replicated at least twice more. Spirochetal multiplication was detected as early as 2 to 7 days after inoculation in cultures incubated at 37°C and 3 to 14 days when cultures were incubated at 33°C. Spirochetes could not be detected in noninoculated cultures or in certain cultures inoculated with fewer than five organisms. Spirochetes were detected in 10 of 12 cultures that received 10 organisms, 7 of 12 cultures that received 5 organisms, and 5 of 12 cultures receiving a single organism. Doubling time during log-phase growth was 10 to 11 h at 37°C and 12 to 13 h at 33°C. Spirochetes generally attained final densities exceeding 10^8 and occasionally 10^9 per ml. These spirochetes grew similarly in microtiter plates. We concluded that the experimental medium supports the multiplication of a single spirochete and that as many as a billion spirochetes per milliliter of medium may develop.

Isolation from ticks and vertebrate tissues. Deer ticks were collected at several sites where the agent of Lyme disease is intensely transmitted, Ipswich, Great Island, and Nantucket in Massachusetts (June and November 1991), and adults were collected at Spooner, Wis. (October 1991). We then compared the sensitivity of diagnosis by culture in the experimental medium with that of a standard immunofluorescence test utilizing monoclonal antibody H5332 for *B. burgdorferi* in these ticks. Spirochetes were cultured from at least two of five ticks from each site that tested positive by immunofluorescence. No spirochetes were detected by culture of samples from ticks that tested negative by immunofluorescence. Thus, this experimental medium appears less sensitive than our immunofluorescence test for detecting infection in ticks but is suitable for regularly isolating Lyme disease spirochetes from field-collected ticks.

The suitability of the experimental medium for detecting spirochetal infection in mouse skin was then evaluated. Tissue from the ears of white-footed mice (fed upon by experimentally infected deer ticks) was placed in wells of microtiter plates containing the experimental medium but no antibiotics. Spirochetes developed in six of nine cultures after 14 days of incubation. Contaminating microorganisms, which developed in four of these cultures, interfered with observations for the three cultures in which spirochetes

could not be detected. When the medium was supplemented with antibiotics, we detected spirochetes in cultures from eight of the nine infected mice. None of these nine cultures were contaminated. Spirochetes were similarly cultured from skin samples removed from the backs of rabbits and mice that had been fed upon by infected ticks (4 to 12 months prior to biopsy). The identity of these spirochetes was confirmed as *B. burgdorferi* on the basis of their reactivity to fluorescein isothiocyanate-labeled monoclonal antibody H5332. We concluded, therefore, that this experimental medium is sufficiently sensitive and selective for reliable diagnosis of infection in natural reservoirs and that addition of antibiotics enhances the diagnostic utility of the medium.

We also tested the suitability of this medium for definitively diagnosing human Lyme disease. An experienced physician removed a 2-mm sample of tissue from the leading edge of an expanding erythema migrans lesion on a human patient using sterile technique and incubated it in 2 ml of the antibiotic-free experimental medium. The resulting culture of Lyme disease spirochetes remained noncontaminated; spirochetes were detectable by dark-field microscopy within 7 days of incubation, and they reacted with monoclonal antibody H5332. This observation establishes the potential of the experimental medium for diagnosing human infection by the agent of Lyme disease.

Infectivity of cultured spirochetes. The infectivity of spirochetes maintained in the experimental medium was tested. Thus, N40 spirochetes that had been maintained in the natural cycle of transmission were reisolated from a mouse and maintained in the experimental medium through 10 serial passages. Inocula of 5×10^5 spirochetes were injected intradermally into each of five mice (strain CD-1). To confirm infection in each animal, larval ticks were permitted to feed upon mice 3 to 4 weeks after infection and were crushed and examined by dark-field microscopy after they molted to the nymphal stage. Infection was thereby confirmed by dark-field microscopy as well as direct fluorescent-antibody assay for each of the five mice. Some of these infected ticks were permitted to feed on noninfected mice, and the resulting infection was similarly confirmed. These observations establish that spirochetes remain infective to mice and to ticks when maintained through a series of passages in the experimental medium.

Stability. The stability of the experimental medium was tested by assessing spirochetal growth dynamics following prolonged storage at various temperatures. Toward this end, aliquants of the experimental medium were stored in the dark for more than 8 months at −80, −20, 4, 33, and 37°C. Samples were then thawed, supplemented with rabbit serum that had been stored at −20°C, inoculated with one to five spirochetes per culture, and incubated at 37°C. Medium stored at −20 and −80°C supported growth of such small inocula to densities exceeding 10^8 spirochetes per ml within 3 weeks in seven of nine cultures. Although storage at 4°C affected neither the medium's ability to support growth of small inocula nor the final density of spirochetes, the time required for attaining detectable and final densities was extended to 4 to 10 weeks. No gross differences in spirochetal morphology or motility were observed after incubation in medium stored between −80 and 4°C. Spirochetes grown in fresh medium and in medium stored for prolonged periods reacted similarly with our battery of immunological reagents; we did not further determine whether prolonged storage of BSK-H medium induced antigenic change in these spirochetes. No growth of spirochetes in medium that had been stored at 33 or 37°C for 2 months was detected. Thus, frozen experimental medium remains stable for at least 8 months.

We then determined whether Lyme disease spirochetes retain viability when frozen in the experimental medium. Cultures of each isolate tested were grown to densities of 10^6 to 10^8 organisms per ml, transferred to 2-ml cryostorage tubes, and frozen at −80°C for as long as 8 months. Triplicate tubes were then transferred every 2 months from −80°C storage and incubated at 37°C. Spirochetal growth was detectable within 2 to 7 days for all 12 cultures. Morphology and growth kinetics of these spirochetes remained normal, and their viability was confirmed by passage into mice. Thus, the experimental medium serves as a useful cryostorage medium for these spirochetes.

Growth of other spirochetes. Finally, the suitability of the experimental medium for growing diverse strains of Lyme disease spirochetes and for borreliae transmitted by soft ticks (Table 1) was evaluated. Thus, inocula of 5 to 10 organisms, in triplicate, were placed in individual culture tubes, and spirochete density was recorded after 2 to 3 weeks. All cultures developed to a density of at least 10^8 organisms per ml. This demonstrated that the experimental medium is suitable for the growth of relapsing fever as well as Lyme disease spirochetes.

DISCUSSION

Although particular batches of BSK-II medium, prepared according to the original recipe, fulfill the ideal criteria (2) for a satisfactory spirochete growth medium, other batches frequently have proven to be unsatisfactory. Proteinaceous components, such as BSA (4), are highly variable and profoundly affect the quality of the final product. The source and quality of the serum supplement are also a critical factor. Indeed, one of six batches of rabbit serum purchased from several different suppliers contained antispirochetal immunoglobulin G detectable by ELISA; this sample failed to support spirochetal growth. Antispirochetal antibody, perhaps because of a response to commensal treponemes or to *Treponema cuniculi* (12), may inhibit the growth of borreliae. Our modified medium, however, eliminates this uncertainty. The modifications that we describe facilitate preparation of bulk lots (>300 liters per lot) of prescreened medium that are stored for future use, and the current commercial availability of these preparations facilitates distribution. Nonspecialized laboratories, thereby, can circumvent the labor- and cost-intensive requirements involved in preparing and prescreening batches of media. Direct comparisons of research results using spirochetes maintained in standardized medium are now possible.

Diverse tick-borne spirochetes develop in this standardized medium. This medium supports the growth of the argasid-tick-borne hematotrophic spirochetes as well as variously adapted dermatotrophic *Ixodes* sp.-borne Lyme disease spirochetes. Indeed, small inocula (approaching a single organism) of each of these infectious agents develop rapidly to densities approaching 10^9 organisms per ml of medium. During their log phase of growth, inocula double in this medium in about half a day, a period which is similar to the 7.6-h estimate derived for natural multiplication of Lyme disease spirochetes in replete larval deer ticks (7). Culture-derived as well as vertebrate- or tick-derived isolates appear to grow equally well in this medium. Thus, this medium appears to satisfy a variety of spirochete culture requirements.

The availability of this medium may facilitate clinical

diagnosis of Lyme disease. Diagnosis of human infection by the agent of Lyme disease generally is inferential, resting on serological and clinical findings (8). Definitive diagnosis would require demonstration of the pathogen, and this has recently been recommended as a criterion for diagnosis (5). Clearly, direct recognition of spirochetal pathogens provides a greater level of diagnostic certainty than does the more inferential process of serological diagnosis, and the ready availability of a standardized culture system is prerequisite to this process. A biopsy system for human skin, as suggested by the mouse ear punch system of Sinsky and Piesman (9), may provide a clinical model for such a diagnostic routine.

The medium retains virtually all of its growth-supporting properties when frozen for half a year or more, and it retains some of these properties when chilled. This stability provides the basis for standardization of the medium. Large batches may thereby be screened and stored for later use, circumventing the need for frequent preparation and screening of small-volume batches. In addition, this medium is appropriate for cryostorage of spirochetes, even without additives. When thawed, such spirochetes grow rapidly and remain infective. This standardized medium can be stored.

Two large batches of the experimental medium already have been produced and evaluated. Pretested rabbit serum (Sigma R-7136) similarly is available for use as a supplement. This modified medium has been designated BSK-H to indicate its basic similarity to BSK-I and BSK-II and has become available commercially (Sigma B-3528). In addition to its various experimental uses, this product may ultimately satisfy clinical requirements for an in vitro diagnostic reagent.

ACKNOWLEDGMENTS

This study was supported in part by grant AI29724 from the National Institutes of Health.

Sera and components required for the formulation and testing of experimental media were provided by Sigma Chemical Co., Cell Culture division. We thank E. Lerner of the Massachusetts General Hospital, Boston, Mass., and A. Kiszewski of the Harvard School of Public Health for providing human biopsy specimens.

REFERENCES

1. **Anderson, J. F., P. H. Duray, and L. A. Magnarelli.** 1987. Prevalence of *Borrelia burgdorferi* in white-footed mice and *Ixodes dammini* at Fort McCoy, Wis. J. Clin. Microbiol. **25:** 1495–1497.
2. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
3. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Menach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216:**1317–1319.
4. **Callister, S. M., K. L. Case, W. A. Agger, R. F. Schell, R. C. Johnson, and J. L. E. Ellingson.** 1990. Effects of bovine serum albumin on the ability of Barbour-Stoenner-Kelly medium to detect *Borrelia burgdorferi*. J. Clin. Microbiol. **28:**363–365.
5. **Centers for Disease Control.** 1990. Lyme disease. Morbid. Mortal. Weekly Rep. **39:**19–20.
6. **Donahue, J. G., J. Piesman, and A. Spielman.** 1987. Reservoir competence of white-footed mice for Lyme disease spirochetes. Am. J. Trop. Med. Hyg. **36:**92–96.
7. **Piesman, J., J. R. Oliver, and R. J. Sinsky.** 1990. Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). Am. J. Trop. Med. Hyg. **42:**352–357.
8. **Schwan, T. G., W. J. Simpson, and P. A. Rosa.** 1991. Laboratory confirmation of Lyme disease. Can. J. Infect. Dis. **2:**64–69.
9. **Sinsky, R. J., and J. Piesman.** 1989. Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. J. Clin. Microbiol. **27:**1723–1727.
10. **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.
11. **Telford, S. R., III, and A. Spielman.** 1989. Enzootic transmission of the agent of Lyme disease in rabbits. Am. J. Trop. Med. Hyg. **41:**482–490.
12. **Turner, T. B., and D. H. Hollander.** 1957. Biology of the treponematoses. World Health Organization, Geneva.