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in Barbour-Stoenner-Kelly Medium
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Barbara Johnson-Winn

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COMPARATIVE GROWTH OF BORRELIA BURGDORFERI IN BARBOUR-STOENNER-KELLY II MEDIUM AND MODIFIED MEDIUM PREPARATIONS

By

Tracy William Smith

A THESIS

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ABSTRACT

COMPARATIVE GROWTH OF BORRELIA BURGDORFERI
IN BARBOUR-STOENNER-KELLY II MEDIUM
AND MODIFIED MEDIUM PREPARATIONS

By

Tracy William Smith

Lyme disease, caused by the spirochete Borrelia burgdorferi, is transmitted through the bite of an infected tick. Culturing the spirochete in improved Barbour-Stoenner-Kelly (BSK II) medium from infected rodent reservoirs and tick vectors is a consistent source of viable organisms. But continuous culturing of B. burgdorferi isolates in BSK II causes physical cell changes resulting in the loss of infectivity to rodents.

The main objective of this study was to improve the growth of B. burgdorferi in vitro. Various modifications to BSK II medium were used independently and together to culture low passage B. burgdorferi isolates. These modifications included replacing serum and albumin ingredients with the corresponding human components. Fetal bovine serum and an albumin mixture were also tested. Spirochete concentrations were measured with a Neubauer counting chamber. Culture tubes were inoculated with very low concentrations for recovery comparisons. BSK II medium variants prepared with human albumin provided accelerated growth rates and facilitated recovery of B. burgdorferi.

To my loving wife Marci,
for fulfilling more family responsibilities
which gave me additional time for research and thesis completion.

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INTRODUCTION

A multisystem disease, initially appearing as a migrating rash, affected the residents of Lyme, Connecticut in the late 1970's. Several patients had experienced tick bites and a surveillance program correlated the incidence of disease with the distribution of Ixodes ticks (Steere and Malawista 1979). In 1982, Dr. Willy Burgdorfer discovered a spirochete in the Ixodes dammini tick and suggested the spirochete caused "Lyme disease", a name that reflected the Lyme, Connecticut focus (Burgdorfer et al. 1982). His hypothesis was confirmed when other researchers isolated the spirochete from various tissues of patients with Lyme disease (Benach et al. 1983, Steere et al. 1983). The spirochete was formally named Borrelia burgdorferi (Johnson et al. 1984a). In 1984, Dr. Alan Barbour successfully cultivated B. burgdorferi (Barbour 1984) in a medium that was a modified version of Dr. Herbert Stoenner's derivation (Stoenner et al. 1982) from a formula originally tested by Dr. Richard Kelly on other Borrelia species (Kelly 1971 and 1976). The modified medium was named after these three researchers: Barbour-Stoenner-Kelly (BSK). Further improvements made by Dr. Barbour resulted in the medium nomenclature, BSK II.

BSK II is currently the standard medium used to culture B. burgdorferi; however, researchers recognize that the medium has some serious shortcomings. The spirochetes cannot be maintained indefinitely in vitro. After several passages, the spirochetes undergo morphological cell changes, loss of plasmids, and loss of infectivity to rodents.

This study attempted to improve the recovery and maintenance of B. burgdorferi cultured in BSK II medium through a series of

substitutions to the medium ingredients. Comparing spirochete population doubling times was the primary evaluation tool. Select medium variants were assayed for recovery by parallel inoculation with a limiting dilution series. Maintenance was appraised by observing changes in generation times and cell configurations. The results yielded a promising albumin substitution that should serve to enlighten the nutritional requirements of B. burgdorferi.

LITERATURE REVIEW

Lyme borreliosis is a tick-borne zoonosis with humans as incidental hosts. The etiologic agent of North American Lyme disease is a spirochete, Borrelia burgdorferi which was isolated by Dr. Willy Burgdorfer from an Ixodes dammini tick (Burgdorfer et al. 1982 and Burgdorfer 1984). In 1993, the tick species I. dammini was reassigned (conspecified) into the I. scapularis species (Oliver et al. 1993). Lyme borreliosis also exists in Europe and Asia. The disease initially appears as a small papule at the tick bite site. Spirochete migration through the skin usually produces an expanding rash called erythema migrans. Left untreated, the disease progression can be debilitating but early antibiotic treatment is usually successful.

Within the last five years, researchers have uncovered a variety of new information that often seems contradictory. Some of the contradictions have resulted from variations in research methods. But most of the differences are due to the complex and variable nature of B. burgdorferi which is capable of altering its own phenotypic and genotypic profiles by mechanisms that are just being recognized. After approximately 15 passages in vitro, B. burgdorferi undergoes morphologic and antigenic changes, plasmid loss, and loses the ability to infect rodents. Areas of scientific investigation include in vitro cultivation, maintenance of infectivity, genetic and molecular characterization, vector competence, transmission, host susceptibility, disease pathology, clinical detection, antibiotic treatment, and reducing the risk of acquiring infection. A human vaccine has completed preliminary phase I trials (Keller et al. 1994).

Borrelia burgdorferi bacteriology

B. burgdorferi belongs to the order Spirochaetales, of which three genera contain human and animal pathogens: Treponema, Leptospira, and Borrelia (Johnson, R. et al. 1984b). Of the pathogenic spirochetes, only the Borrelia species are transmitted by ticks (Barbour and Garon 1988, Schmid 1989). Of the borreliae, B. burgdorferi is the longest measuring 10-30 μm, the narrowest at 0.2-0.38 μm diameter, the most loosely coiled with a mean wavelength of 2.2-3.3 μm, and has the fewest flagella between 7-12 per organism compared to 15-30 flagella in other spirochetes (Hovind-Hougen et al. 1986, Johnson, R. et al. 1984a, Kelly 1984, Steere 1989).

Cross sections of B. burgdorferi reveal a protoplasmic cylinder containing the organelles, surrounded by a peptidoglycan layer. A cytoplasmic membrane (CM) covers the peptidoglycan layer. Periplasmic flagella (or endoflagella) are attached subterminally to each end of the protoplasmic cylinder, wrap around the CM, and extend toward the opposite end of the cell cylinder. Flagella from one end overlap the flagella extending from the opposite end. An outer membrane (OM) envelopes the flagella and cytoplasmic membrane (Barbour and Hayes 1986, Goldstein et al. 1994, Radolf et al. 1994, Reik 1991). Similar to other Gram negative bacteria, the OM contains a lipopolysaccharide (LPS) layer that has an endotoxin-like activity (Beck et al. 1985, Fumarola et al. 1986, Habicht et al. 1986). Linear bodies observed within the OM by freeze-fracture electron microscopy, were perpendicular to the axis of the cylinder suggesting a structural function (Radolf et al. 1994). The OM comprises 16% of the dry weight of the spirochete (Coleman et al. 1986). B. burgdorferi sheds membrane vessicles (also referred to as blebs) of various sizes (Barbour and Hayes 1986, Dorward et al. 1991, Garon et al. 1989, Radolf et al. 1994). The blebs have contained extrachromosomal DNA (Garon et al. 1989) and have also contained some of the major antigenic outer surface proteins (Shoberg and Thomas 1993). Shedding blebs may help alter the immunogenic profile of the spirochete.

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B. burgdorferi divides by transverse fission within 7-12 hours but may take up to 20 hours (Barbour 1984, Pollack et al. 1993, Preac-Mursic et al. 1991, Williams and Austin 1992). The spirochetes swim with a spiral, corkscrew-like, planar waveform motion (Goldstein et al. 1994), viewed by either phase contrast or darkfield microscopy. The spirochetes stain weakly Gram negative. Better visualization has been reported with an eosin-thiazine (Hemacolor) stain or silver impregnation although neither staining method is specific (Aberer and Duray 1991). A B. burgdorferi-specific immunohistochemical staining method produced intensely red spirochetes but the spirochetes were visualized in only 39% of the tissue specimens that were positive by culturing (Lebech et al. 1995). B. burgdorferi is beta-hemolytic (Williams and Austin 1992), microaerophilic, catalase and peroxidase negative, and superoxide dismutase positive. The spirochete ferments glucose to lactose via the Embden-Myerhof pathway and requires long chain fatty acids and exogenous cholesterol to form cellular lipids (Barbour and Hayes 1986, Johnson, R. et al. 1984a). N-acetylglucosamine is incorporated directly into the peptidoglycan layer (Beck et al. 1990).

B. burgdorferi, strain B31, was reported by Hayes et al. (1983) to have had a bacteriophage virus with capsid that was discovered by electron microscopy. While in the logarithmic growth phase, there was a significant decrease in the number of spirochetes followed by a dramatic increase in the number of spirochetes in the medium. The culture depletion and regeneration was probably not a result of nutrient exhaustion or build-up of toxic substances but may have been caused by a bacteriophage lytic phase (Barbour and Hayes 1986, Hayes et al. 1983). A lysogenic strain of B. burgdorferi isolated from human skin contained two types of bacteriophages induced by the gyrase inhibitor ciprofloxacin (Neubert 1993).

Molecular biology

B. burgdorferi isolates have heterogeneous protein and antigenic profiles. The profiles change after several passages of in vitro culturing. But culturing is required to obtained enough of the

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spirochete material for molecular characterization. B. burgdorferi protein profiles have variable molecular weights and expression intensities (Hu et al. 1992, Jonsson et al. 1992, Simpson et al. 1991b, Wilske et al. 1992). The presence or absence of certain proteins very likely correlates with spirochete virulence and disease pathology patterns (Barbour et al. 1985, Barbour et al. 1986d, Georgilis et al. 1989, Simpson et al. 1991b, Van Dam et al. 1993, Wilske et al. 1988, Zoller et al. 1991). Antigenic variability allows the spirochete to evade immune response systems. The frequency of phenotypic mutations ranged from 10^{-2} to 10^{-4} in low passage B. burgdorferi strains B31 and HB19 (an isolate from human blood) which developed resistance to a battery of specific monoclonal antibodies (Sadzienne et al. 1992). Isolates from humans with chronic Lyme disease lacked some major surface proteins (Wilske et al. 1986). A unique B. burgdorferi strain 25015, isolated from an I. scapularis tick, was infectious but did not cause carditis or arthritis in laboratory rats or mice. Strain 25015 was frequently isolated from normal joint tissues, had altered surface proteins, and changes in immunoreactivity patterns (Anderson et al. 1990a).

Major outer surface proteins (Osp), OspA (30 to 32 kDa), OspB (33 to 36 kDa), and OspC (21 to 22 kDa), were detected from most B. burgdorferi isolates but expression varied (Barbour 1989b, Barbour and Hayes 1986, Schwan et al. 1993, Wilske et al. 1986). Recently OspD (28.4 kDa; Norris, S. et al. 1992), OspE (19.2 kDa), and OspF (26.1 kDa), have been characterized (Lam et al. 1994). Expression of particular outer surface proteins may depend on temperature and the particular host of the spirochetes. While in an unfed tick, B. burgdorferi produces OspA surface proteins but does not express OspC (Ewing et al. 1994, Schwan et al. 1995). Days after the tick has attached to a warm blooded host and has begun blood feeding, the spirochetes produced OspC. B. burgdorferi cultured in vitro between 32-37°C produced OspC but at 24°C very little OspC was produced. Incubation of infected ticks at 37°C did not stimulate OspC production (Schwan et al. 1995). Expression of OspC may enhance infectivity or be required for transmission to the mammalian host.

In mammals, early antibody response to B. burgdorferi infection has been directed against several antigens but may be strongest against the OspC antigen (Brunet et al. 1995, Padula et al. 1994, Schwan et al. 1995). When infecting mammals, expression of OspA proteins may be temporarily stopped (Golde et al. 1994, Schwan et al. 1995) Expression of OspA increased with repeat in vitro culturing (Wilske et al. 1986). Immunoreactive OspA antigens were expressed more consistently than immunoreactive OspB antigens among isolates from humans (Berger et al. 1985) and ticks (Lane and Pascocello 1989, Schwan et al. 1993). An extensive study mapped rabbit and mouse monoclonal antibody binding sites of an OspA fusion protein (Schubach et al. 1991). The genes encoding both OspA and OspB are located on a linear plasmid and transcribed as a single unit, from a single promoter (Barbour and Garon 1988, Howe et al. 1986). The *ospA* and *ospB* gene sequences are similar (Bergström et al. 1989). Expression of a 39kDa protein (p39) may be more conserved since host immune responses have consistently and persistently reacted with the p39 antigen (Brunet et al. 1995, Golde et al. 1994, Johnston et al. 1992, Roehrig et al. 1992, Schwan et al. 1993, Simpson et al. 1991a). A 60 kDa antigen of unknown function is strongly immunogenic and cross-reactive with an equivalent antigen from a wide range of remotely related bacteria (Hansen et al. 1988a). Three other cross-reactive antigenic determinants (19, 22, and 72 kDa) are shared with other bacteria (Coleman and Benach 1987 and 1992).

Other cellular components include a flagellar antigen (41 kDa; Barbour et al. 1986), cell surface glycoproteins (Sambri et al. 1992), an uncharacterized lipopolysaccharide layer (8 to 11 kDa; Beck et al. 1985, Cinco et al. 1991), four heat shock proteins (Cluss and Boothby 1990, Coleman and Benach 1987), and a peptidoglycan layer containing N-acetylglucosamine, alanine, glutamic acid, glycine, and ornithine (Beck et al. 1990). Fatty acid methyl ester (FAME) profiles of a variety of B. burgdorferi isolates clustered into three distinct groups (Livesley et al. 1993). Mass spectroscopy identified the presence of five phospholipids: phosphatidylethanolamine, phosphatidic acid, phosphatidylcholine, phosphatidylglycerol, and

phosphatidyldimethylethanolamine. (Dr. Michael Kron, Michigan State University, unpublished data).

Heterogeneous plasmid profiles have been observed (Barbour 1988b, LeFebvre et al. 1990, Schwan et al. 1988a, Schwan et al. 1993, Simpson et al. 1990, Spielman 1988). B. burgdorferi may contain four to nine different plasmids. Some plasmids are supercoiled while others are linear (Hyde and Johnson 1988, Schwan et al. 1988a, Simpson et al. 1990). Critical hereditary information may be carried by more than one plasmid (Barbour and Garon 1988). Loss of infectivity coincided with the loss of a 7.6 kb circular plasmid and a 22 kb linear plasmid (Schwan et al. 1988a).

Although much genetic homology has been identified among B. burgdorferi isolates (Johnson, R. 1984a, Schmid et al. 1984), genetic heterogeneity between isolates from North America and Europe has been demonstrated by plasmid analysis, restriction fragment polymorphisms, and hybridization of nuclear DNA (LeFebvre et al. 1989, Stalhammar-Carlemalm et al. 1990). DNA methylation also differs among isolates but the G + C content for all isolates remains about 29 mol% (Hughes et al. 1992). DNA hybidization of ten isolates from Europe and the United States had a relatedness of 58 to 98% and a divergence of 0.0 to 0.1% (Anderson et al. 1989). B. burgdorferi was 30 to 58% homologous to B. recurrentis (North American relapsing fever borreliae) but only 16% homologous to treponemal DNA and about 1% to leptospiral DNA (Anderson et al. 1989).

Phenotypic and genotypic differences have led to the differentiation of B. burgdorferi sensu lato into three distinct species: B. burgdorferi sensu stricto, B. garinii, and B. afzelii (or group VS461; Baranton et al. 1992) A suggested fourth distinct species B. japonica has only been found in Japan (Masuzawa et al. 1995, Yanagihara et al. 1994). The other three varieties exist in Europe and Asia. The B. burgdorferi sensu stricto group includes all North American and some European isolates but type B31 of B. burgdorferi sensu stricto exists only in North America (Baranton et al. 1992, Bergström et al. 1991, Boerlin et al. 1992, CDC 1992, Marconi and Garon 1992, Rosa et al. 1991, Welsh et al. 1992, Wilske et al. 1991 and 1995). There may be additional subtypes within the

sensu stricto group (Adam et al. 1991, Assous et al. 1994). Clinical manifestations of Lyme disease and immunologic response may be related to genospecies type. While infection with B. burgdorferi sensu stricto spirochetes often lead to rheumatic symptoms, B. garinii group genotypes are associated with neurological complications, and the B. afzelii group spirochetes often cause dermatological manifestations (Anthonissen et al. 1994, Assous et al. 1993, Canica et al. 1993, Demaerschalck et al. 1995, Marconi and Garon 1992, Wilske et al. 1993, Van Dam et al. 1993) European Lyme disease patients may become infected with more than one genotype at one time (Demaerschalck et al. 1995). Reliance on borrelial antigens for serodiagnosis of infection should be constructed from antigens derived from all genotypes that may exist in the geographical area (Assous et al. 1993, Bunkis et al. 1995, Demaerschalck et al. 1995)

Existence in nature

B. burgdorferi spirochetes are maintained in nature by surviving in tick vectors and tick hosts, primarily rodents (Anderson 1989a, Burgdorfer 1989). One mechanism of maintaining B. burgdorferi in nature is through horizontal transmission between vectors and hosts. The spirochete is transmitted from an infected host to an uninfected tick during the tick's blood meal. When infected ticks feed, the spirochetes residing in the tick's midgut tissues are transmitted either by regurgitation from the midgut or in the saliva (Burgdorfer 1989, Ewing et al. 1994, Piesman 1995, Ribeiro et al. 1987). Horizontal transmission promotes disease amplification by spreading spirochetes to uninfected individuals. For example, ticks overwintering with nesting rodents increase the chances of infecting new rodent offspring and newly hatched tick larvae. Transmission to laboratory mice was reduced when the infected ticks were incubated at temperatures above 27°C (Shih et al. 1995b).

Transstadial transmission occurs when ticks infected with a high concentration of spirochetes remain infected through molting

periods. After molting from one life stage to the next, the concentration of spirochetes is greatly decreased (Piesman 1989, Piesman et al. 1990). There may be a critical infection concentration below which the population of spirochetes are unable to survive during molting. Transstadial and transovarial transmission, although not repeatedly demonstrated, was suggested by immunofluorescent assays of infected female Ixodes spp. ticks, their eggs, and larval offspring (Lane and Burgdorfer 1987, Stanek et al. 1986). Contact transmission between housed rodents is controversial (Barthold 1991, Burgess et al. 1986).

An efficient surveillance method of naturally infected wild rodents is by cultivating ear punch biopsies. The animals can be trapped live, sampled once or several times over the course of the field study without endangering the life of the rodent, then tagged, and released. Improved spirochete density in the specimen occurs when the punches are made near the base and center of the ear. Collection of one or more ear punch biopsy specimens per rodent must be performed as aseptically as possible. Swabbing the ear skin with ethanol or betadine solution prior to sampling, greatly reduces the survival of surface contaminants accompanying the sample specimen. After collection, specimen transport, and storage, but immediately prior to culturing, some researchers surface sterilize specimens. Ear punch biopsies have been cleaned again by a dip in 95% ethanol followed by a rinse in a solution of equal volumes of 10% bleach and 95% ethanol (Oliver et al. 1995). Another method of disinfecting whole rodent ear and tail specimens, first soaked them in Wescodyne (Amsco Medical Products, Erie, PA) for 15 minutes, then rinsed with 70% ethanol to remove the Wescodyne, soaked in 70% ethanol for another 15 minutes, and finally rinsed with ethanol again (Campbell et al. 1994). Reducing contamination is paramount when attempting to isolate organisms by culturing but the possible detrimental effects of excess surface sterilization is not known. Spirochetes cultured from ear punch biopsies have been observed within one to two weeks of incubation but rarely may take up to six weeks (Pollack et al. 1993, Sinsky and Piesman 1989).

The distribution of spirochete infections within a field study site are correlated between hosts, ticks removed from hosts, and host seeking ticks collected by dragging. Ticks have been surface sterilized prior to dissection or trituration by dipping them in 3% or 30% (v/v), hydrogen peroxide for one to 15 minutes, followed by a dip in 70% ethanol for two to 15 minutes, a rinse in phosphate buffered saline, and then air dried (Livesley et al. 1994, Schwan et al. 1993). A shorter disinfection protocol washed each tick in 70% ethanol for 10 minutes and then performed three rinses with sterile water for one minute each (Pollack et al. 1993). Spirochetes were recovered from tick saliva collected in a micropipette surrounding the tick mouthparts. Salivation was induced by pilocarpine. B. burgdorferi was grown from saliva volumes of 10 to 20 microliters per tick, inoculated into BSK II medium (Ewing et al. 1994). B. burgdorferi was detected in I. scapularis ticks with an OspA antigen-capture enzyme-linked immunosorbent assay (ELISA) with a reported sensitivity of 30 spirochetes (8 fg). The sensitivity was not affected by the presence of a blood meal (Burkot et al. 1994). Storage of spirochete infected ticks at temperatures above 27°C may be detrimental to spirochete survival (Shih et al. 1995b).

Tick vectors

Ticks flourish in lush woodland habitats. They are more concentrated along the edges of woodland habitats and in grassy meadows in close proximity to woods (Duffy et al. 1994b, Maupin et al. 1991). A landscape survey performed in Westchester County, New York, found most I. scapularis ticks in wooded areas, some lived on the edge of woodlots, less in ornamental vegetation, and very few (2%) came from residential lawns (Maupin et al. 1991). Weather patterns, especially temperature and humidity, geographic features, and host populations all contribute directly to the abundance of ticks and indirectly to the prevalence of infection (Duffy et al. 1994a, Korenberg 1994, Piesman et al. 1987a, Platt et al. 1992, Wilson and Spielman 1985). The most competant vectors of B. burgdorferi are ticks in the I. ricinicus group (in Michigan, I.

scapularis). I. pacificus, the western black-legged tick, is the principle vector on the western coast of the United States (Burgdorfer et al. 1985, Lane and Lavoie 1988).

I. scapularis has a three stage life cycle (larva, nymph, adult) that survives for two years. Although only one blood meal is needed at each stage, the tick may feed on humans at any stage. Immature ticks usually feed on rodents, small mammals, or birds (Anderson 1989a and 1989b, Burgdorfer 1989). Adult I. scapularis ticks usually feed on deer but will feed on other large or medium sized animals (Burgdorfer 1989, Lane et al. 1991, Wilson et al. 1990). Feeding to repletion took about 120 hours for adult female I. scapularis ticks. Transmission of B. burgdorferi during the tick's blood meal requires a lengthy time of tick attachment. Less than 2% of larval I. scapularis ticks that were attached to an infected host for an 8 hour partial blood meal became infected with B. burgdorferi. Infection of a new host is likely only when the tick has been attached for more than 24 to 48 hours (Burgdorfer and Gage 1986, Piesman 1991 and 1993, Piesman et al. 1987b and 1991, Ribeiro et al. 1987, Spielman et al. 1984).

B. burgdorferi growth within ticks is affected by physiological events in the tick's life-cycle metamorphosis. Spirochete concentration dropped 5-fold during the larval to nymphal premolting period. After the nymphs were fed to repletion, the mean density of spirochetes was 30 times higher (61,275 spirochetes/nymph) than the mean density in the larvae. A 10-fold drop occured after the nymphs molted to adults (Piesman 1989, Piesman et al. 1990). B. burgdorferi OspA expression also varied during passage through the tick's life cycle (Burkot et al. 1994). The spirochetes adhere strongly to tick midgut cells (Benach et al. 1987) and may penetrate the gut (Zung et al. 1989). Spirochetes are located in the saliva (Ewing et al. 1994, Piesman 1995), midgut, or distributed systemically via the hemolymph (Burgdorfer 1984, Burgdorfer et al. 1988 and 1989, Ribeiro et al. 1987). The presence of spirochetes in tick salivary glands was facilitated by tick feeding. The incidence of spirochete positive cultures from salivary gland homogenates were increased four-fold when the ticks were

allowed to feed for 72 hours. Spirochete infectivity to laboratory white mice may also have been improved after 60 hours of tick attachment (Piesman 1995). B. burgdorferi has been detected in ticks using polymerase chain reaction (PCR) analysis in addition to culturing (Persing et al. 1990b).

I. ricinus, the sheep tick, is the main European vector (Radda et al. 1986, Schmid 1985). I. persulcatus, the taiga tick, is the main vector in Asia (Anderson 1989b, Burgdorfer 1989). Species of ticks suspected of being inefficient vectors for B. burgdorferi are Amblyomma americanum, Dermacentor variablis, and Haemaphysalis leporispalustris (Anderson and Magnarelli 1984, Lindsay et al. 1991, Magnarelli et al. 1986, Piesman 1989, Rawlings and Teltow 1994). D. occidentalis may be a secondary vector in some areas (Lane and Lavoie 1988). Immature ticks from other genera, particularly D. variabilis, may also become infected when they feed on infected mice (Magnarelli and Anderson 1988b).

Other biting insects

As early as 1939, spirochetal infections were observed in the salivary glands and digestive tracts of naturally infected mosquitoes (Spielman 1988). In the mid-1980's, when a heighten awareness of Lyme disease was sweeping the northeastern United States, B. burgdorferi was reported to have been recovered from horse flies, deer flies, and mosquitoes (Anderson and Magnarelli 1984, Magnarelli et al. 1986). Other than rare individual claims of Lyme disease being contracted by any one of a plethora of biting insects, there has been no scientific reports that suggest any other insects are frequent vectors. Fleas (Orchopeas leucopus), were found to be poor vectors (Lindsay et al. 1991). B. burgdorferi survived only briefly in experimentally infected Aedes mosquitoes (Magnarelli et al. 1987c). Field-collected Aedes spp. mosquitoes were unable to effectively transmit B. burgdorferi to uninfected Syrian hamsters (Magnarelli and Anderson 1988b).

Rodent reservoirs

B. burgdorferi has been isolated from captured wild white-footed mice (Peromyscus leucopus) all seasons of the year including winter when the ticks are inactive (Anderson et al. 1987). A plethora of rodent hosts have been shown to become infected with B. burgdorferi including: deer mice (P. maniculatis), meadow jumping mice (Zapus hudsonius), piñon mice (P. truei), eastern chipmunks (Tamias striatus), bog lemmings (Synaptomys cooperi), California kangaroo rats (Dipodomys californicus), cotton rats (Sigmodon hispidus), dusky-footed wood rats (Neotoma fuscipes), shrews (Blarina brevicauda), and meadow voles (Microtus pennsylvanicus). (Anderson et al. 1985, Lane 1990, Levine et al. 1985, Mather et al. 1989, McLean et al. 1993a, Oliver et al. 1995, Telford et al. 1990, Walker et al. 1994, Zingg et al. 1993).

Once the spirochetes are inoculated into a rodent through a tick bite, the spirochetes reside in the skin for about two days before disseminating (Shih et al. 1992). By three days, the spirochetes have entered the blood stream and spleen (Barthold et al. 1991). Spirochetemia alternates between mild and heavy episodes and persists for at least 30 days (Barthold et al. 1991, Burgdorfer and Schwan 1991). Spirochetes then enter organs, deeper tissues, and have been observed residing within blood vessel endothelial cells, and cardiac myocytes (Pachner et al. 1995). Electron micrographs also revealed low numbers of spirochetes wrapped around collagen fibers (Pachner et al. 1995). In advanced disease cases, B. burgdorferi has been most frequently isolated from the bladder, followed by the spleen, kidney, and heart, and rarely from the blood and urine (Anderson et al. 1986a, Bosler and Schulze 1986, Callister et al. 1989, Goodman et al. 1991, Hofmeister et al. 1992, Schwan et al. 1988b). Recovery from the bladder required an average of six days cultivation in vitro (Callister et al. 1989). Spirochetes have been cultured from one P. leucopus fetus but transplacental transmission is unlikely (Anderson et al. 1987, Mather et al. 1991). It was previously thought that the rodent reservoir did not suffer from the infection (Donahue et al. 1987, Levine et al. 1985) but a

survey of captured wild white-footed mice attributed motor abnormalities and neurologic damage in the mice to B. burgdorferi infection (Burgess et al. 1990). Naturally infected adult mice develop a rapid, strong immune response with additional antigens recognized over time (Brunet et al. 1995). Rodent reservoir antibodies were more often directed against the p39 antigen than OspA, OspB, OspC or flagellar antigens (Brunet et al. 1995, Golde et al. 1994, Simpson et al. 1991a).

Experimental infections in several species of laboratory rodents have been introduced by intraperitoneal injection (Barthold 1991, Barthold et al. 1990, Moody et al. 1990). Laboratory mice injected with B. burgdorferi produced a strong, early antibody response to the OspA antigen (Barthold et al. 1991). The use of natural vectors in experimental infections provides results more valid than unnatural inoculations. Unnatural injection nullifies host-vector biological interactions, spirochete dose rate, and delivery mechanisms (Randolph and Nuthall 1994). Mice infected by syringe injection were not as infective to naive ticks as mice naturally infected by infected ticks (Gern et al. 1993). Spirochete-free ticks also provide xenodiagnosis of spirochetemic rodents.

Other animal hosts

White-tailed deer (Odocoileus virginianus) are the principle host of adult I. scapularis ticks and also have been shown to carry antibodies to B. burgdorferi (Bosler et al. 1984, Gill et al. 1993, Lane and Burgdorfer 1986, Magnarelli et al. 1986). Deer and rodents are not the only animals to harbour B. burgdorferi spirochetes. Borreliosis has been reported in naturally infected dogs (Bosler et al. 1988), bears (Kazmierczak et al. 1988), cattle (Burgess 1988, Osebold et al. 1986, Parker and White 1992), coyotes (Burgess and Winberg 1989), a fox (Isogai et al. 1994), horses (Bosler et al. 1988, Burgess 1988, Parker and White 1992), rabbits (Tälleklint and Jaenson 1993), sheep (Fridriksdottir et al. 1992), wolves (Thieking et al. 1992), and other small to medium sized animals (Fish and Daniels 1990, Smith et al. 1993). At least 31 mammalian species

and 49 species of birds have been found to be parasitized by the ticks (Anderson 1988 and 1989a, Magnarelli and Stafford 1992, Nakao et al. 1994). Laboratory rabbits became hosts when experimentally infected (Benach et al. 1987, Kornblatt et al. 1984, Moody et al. 1990). Dogs suffering from the effects of Lyme disease have received much public and veterinary attention. Dogs may develop fever, anorexia, or fatigue but lameness or other disorders associated with the limbs or joints are the most common symptoms (Kornblatt et al. 1985, Magnarelli et al. 1988c, Appel et al. 1993). A survey of dog sera may be useful to predict areas at risk for human Lyme disease (Daniels et al. 1993, Lindenmayer et al. 1991, Rand et al. 1991). Immunofluorescent or enzyme-linked immunosorbent assays are available for screening dog sera for the presence of antibody directed against B. burgdorferi (Falco et al. 1993, Lindenmayer et al. 1990, Magnarelli et al. 1987b). Cultivating ear punch biopsies from dogs that were experimentally infected by heavy tick feeding produced spirochete cultures from all ten test dogs. Spirochetes were recovered from ear biopsies taken between 6 and 17 days following final tick feeding but biopsies taken 80 days post feeding were all negative (Bosler et al. 1992).

B. burgdorferi has been isolated from the blood of a song sparrow (Melospiza melodia), the liver of a veery (Catharus fucesens), and from I. scapularis larvae feeding on other birds (Anderson et al. 1986b, McLean et al. 1993b). B. burgdorferi was transmitted from birds to Syrian hamsters by ticks (Anderson et al. 1990b). Infected ticks may become attached to birds and be distributed to new local habitats or more distant areas (Anderson et al. 1990b, Matuschka and Spielman 1986).

Disease control strategies

Pathogens of Ixodes ticks that may be used as biological control methods include fungi, protozoa, a phorid fly, carabid beetles, and chalcidoid wasps (Jaenson et al. 1991). The chalcid wasp (Hunterellus hookeri) oviposits into certain tick species eventually killing the tick (Mather et al. 1987a). Helmeted

guineafowl feed on ticks, primarily adults. Deer tick numbers were reduced within penned enclosures containing the fowl. However, deployment of guineafowl alone was deemed not effective in significantly reducing the risk of human Lyme disease cases (Duffy et al. 1992).

Chemical spray applications are useful for protecting a small yard or house for a relatively short period during the tick season. Large scale applications threaten the health of many organisms, fail to adequately penetrate dense vegetation, and were only effective against nonfed ticks that climbed the foliage questing for a new host (Schulze et al. 1987). A novel approach to chemical application distributed weather-resistant tubes containing cotton batting treated with an acaricide (permethrin) into mice habitats (Mather et al. 1987b). The rodents collected the treated cotton for nesting material. The treatment reduced rodent parasitism and the rodents showed no ill effects over short term observation. Nymph populations were reduced and the percentage of infected nymphs was reduced. The risk of human infection was reduced by an estimated 82%. A repeat study of this method over a larger land area was so successful that commercial manufacturers marketed the device (Mather 1988, Spielman 1988). A similar method employed baited tubes that doused rodents with diazinon when they passed through the tubes (Sonenshine and Haines 1985). Baits containing systemic acaricides have been used to control ticks on domestic animals but not wild animals (Drummond et al. 1981, Lancaster et al. 1982). Acaricide impregnated discs placed on deer were not effective (Spielman 1988).

Clearing tick habitats by mowing or burning were ineffective at maintaining a significant reduction in tick numbers (Mather et al. 1993, Wilson 1986). Reducing host numbers is impractical but was performed in a deer reduction study (Wilson et al. 1984). Reduction in available deer hosts reduced tick density but did not eliminate the tick population (Duffy et al. 1994a, Wilson et al. 1984).

Humans and house pets should avoid entering suspect areas during the late spring through summer tick season (June to August). Someone that must be exposed to possible tick-infested sites should

wear protective clothing (long pants with taped cuffs for example). Deet and permethrin, sprayed on clothing repels ticks. Further precautions should be taken when removing protective clothing. Carefully inspect all skin surfaces for tick attachment, especially hairy areas. If a tick is located it should be removed with tweezers but not with fingers. Avoid squeezing the tick which causes regurgitation of gut contents (Anderson 1989d, Lane 1989, Needham 1984, Schreck et al. 1986, Stafford 1989).

Experimental vaccinations and immunizations

A phase I clinical trial among humans examined the immunogenicity of a two vaccine formulas (with or without adjuvant) containing recombinant OspA antigen. Conducted with 36 volunteers, the testing was randomized, double blind, and placebo-controlled. After two intramuscular injections 30 days apart, the test groups produced significant titers of IgG antibody directed against OspA that inhibited the growth of B. burgdorferi cultured in medium held in a sealed microtiter plate. Some local and systemic adverse reactions also resulted. Larger studies to optimize dosage and evaluate efficacy are in progress (Keller et al. 1994).

An active immunization prepared from a high concentration of inactivated whole B. burgdorferi cells protected hamsters from infection for at least 30 days. Passive immunization with B. burgdorferi antiserum provided strain specific protection but not full cross strain protection (Johnson, R. et al. 1986 and 1988). Recombinant OspA protein produced in Escherichia coli and injected into laboratory mice, induced antibody production and protected the mice from being challenged with several but not all strains of B. burgdorferi (Fikrig et al. 1990 and 1992a, Simon et al. 1991, Telford III 1993). Spirochetes were killed within infected ticks by the ingestion of immune antibodies while feeding on mice vaccinated with recombinant OspA or OspB (Fikrig et al. 1992b and 1995, Telford et al. 1993), recombinant OspE or Osp F (Nguyen et al. 1994), or immunized with pooled anti-spirochetal serum (Shih et al. 1995a).

A vaccine for dogs, B. burgdorferi bacterin (Fort Dodge Laboratories, Fort Dodge, Iowa), was given a provisional license by the USDA in 1990 for distribution. But it wasn't until 1992 that the manufacturer published results of experimental trials. The vaccine was synthesized from an inactivated B. burgdorferi strain isolated from the eastern United States. There have been no major side-effects, other than a short term fever, associated with the vaccination. The vaccine was shown to be immunogenic, eliciting a strong antibody response primarily directed against the OspA and OspB antigens. However, it did not confer complete protection among trials conducted by the manufacturer (Chu et al. 1992). Five of 30 (17%) of the vaccinated dogs developed spirochetemia after being injected with B. burgdorferi cultures, compared to 15 of 24 (63%) of the control dogs. Legitimate criticisms of the manufacturer's experimental methodology revealed test bias and doubtful results (Kazmierczak and Sorhage 1993).

The canine Lyme disease vaccine failed to establish long term immune protection in hamsters. Peak antibody response occurred three to five weeks after the primary vaccination and declined by six weeks. A booster, given three weeks after the primary, extended peak response to ten weeks. Vaccinated hamsters contracted Lyme arthritis when challenged by B. burgdorferi isolates other than the isolate used to prepare the vaccine. Further antibody response characterizations concluded that the best protection was conferred by IgG antibodies rather than IgM antibodies (Jobe et al. 1994).

Epidemiology of Lyme disease

Lyme disease has been reported in Africa, Asia, Australia, Europe, and North America (Barbour 1988a, Schmid 1985). Correlations between Lyme disease incidence and I. scapularis distribution across the United States have been documented since 1979 (Steere et al. 1979). In the United States, three regions are endemic for Lyme disease. The first region covers the east coast from Maryland to Massachusetts. The second region, located in the north central U.S. includes Minnesota, Wisconsin, and a western

portion of Michigan's upper peninsula. The third region covers the west coast from California to Oregon and western Nevada. Additional cases of Lyme disease have been reported in 47 of the United States (CDC 1993a and 1993b).

Lyme disease is the most common arthropod-borne infection in the U.S. and has been reported from 43 states (Agger et al. 1991, Barbour 1988a, CDC 1989, Ciesielski et al. 1989, Tsai et al. 1989). The number of human cases of Lyme disease in the United States was 7,943 in 1990, 9,465 in 1991 (CDC 1992), and 9,677 in 1992 (CDC 1993a). Of the 4,999 cases reported by mid-1992, 80% occurred in the east coast region (CDC 1992). The first isolations of B. burgdorferi by culturing skin punch biopsies from human patients in Michigan occurred in 1992 (Stobierski et al. 1994).

Clinical presentation and pathology

Lyme disease is usually transmitted to humans during the bloodmeal of certain Ixodid ticks. Transmission of the spirochete from an infected tick to a new host requires one to two days attachment (Berger et al. 1995, Piesman et al. 1987b, Ribeiro et al. 1987). After the infected tick bite, a papule forms at the bite site. Within approximately one month (median 7-10 days) the patient will usually develop a patchy rash called erythema migrans (EM). The presentation of this rash is the hallmark of early indication of Lyme disease but may occur less frequently than previously thought (Berger et al. 1995, Ley et al. 1994, Nadelman and Wormser 1995). EM appears as solid red rash zones somewhat similar to a patchy or local intense sunburn. The inflammatory zone expands in reaction to the outward migration of the spirochetes. Eventually the center of the lesion turns pale with hot, red outer margins which gives the rash a bulls-eye appearence. Two to three weeks later the entire lesion fades. This is the preferred time to begin antibiotic treatment, with varying aggressiveness, which gives the patient an excellent chance of complete recovery. To some physicians, EM presentation warrants immediate antibiotic treatment despite possible seronegative results of Lyme disease testing (Berg et al.

1991, Duray and Steere 1988, Zoschke 1992). If left untreated, the EM lesions will disappear but the spirochetes may persist in the skin six months after the skin clears (Kuiper et al. 1994). Even though the the risk of infection from a tick bite in an area hyperendemic for Lyme disease was very low (zero when tick attachment was less than 24 hours), prophylactic antibiotic treatment was suggested after a tick bite (Berger et al. 1995). After attaining similiar results among a much larger group of human subjects, other researchers determined the risk of infection was so low that the potential adverse side effects of treatment outweighed the benefits and therefore did not recommend prophylactic treatment (Shapiro et al. 1992).

An active cellular immune response may preceed the humoral response (Dattwyler and Thomas 1986, Dattwyler et al. 1988, Golightly et al. 1988). The flagellar and OspA antigens stimulate lymphocytes, especially T cells, to proliferate (Benach et al. 1988, Dattwyler et al. 1988). Monocyte response increases as the disease progresses. Most monocyte activity occurs in the joint fluid of arthritis complicated cases (Sigal et al. 1986). Recent evidence points to early production of IgM antibodies that become complexed with the spirochetal antigens, thereby reducing clinical antibody detection without disassociating the complexes (Schutzer et al. 1994).

Initial antibody (IgM) production was thought to be directed against OspA, flagellar, and other B. burgdorferi antigens. Then subsequent antibody production (IgG) late in the disease was primarily directed against OspA, OspB, and protein C (Coleman and Benach 1987, Craft et al. 1986, Hechemy et al. 1988). Recent evidence suggests that early antibody response is directed against OspC (Aguero-Rosenfeld et al. 1993, Dressler et al. 1993, Fung et al. 1994, Padula et al. 1993 and 1994). B. burgdorferi is resistant to complement activity unless a specific IgG antibody is present. Binding of the specific antibody to the spirochete, alters the outer cell membrane allowing attachment of the complement membrane attack complex (Kochi et al. 1991). The antigenic variability of B.

burgdorferi significantly affects antibody production of the immune response (Craft et al. 1986, Dattwyler et al. 1989).

The spirochetes penetrate the subendothelium into the blood and lymph vessels. As patient antibody titers rise to detectable levels, the spirochetes move from the blood into various tissues (Magnarelli 1988, Steere et al. 1983). Early secondary manifestations include: flu-like symptoms, fever, muscular pain, severe headache, and swollen lymph nodes in the region of the initial infection (Rahn 1991, Sigal 1990). B. burgdorferi has been shown to penetrate and reside intracellularly in human endothelial cells in vitro (Comstock and Thomas 1989, Ma et al. 1991, Szczepanski et al. 1990). Prolonged antibiotic therapy is needed to combat the disseminated intracellular infections that give Lyme disease its subtle but persistent nature. Three weeks to six months after the initial tick bite, secondary symptoms develop due to the invasion of the reticuloendothelial system, deeper tissues, and organs (Duray 1989a, Steere 1989).

Profound fatigue and muscle pain persist throughout the course of the disease. Lyme disease has been associated with fibrositis (or fibromyalgia) which describes general fatigue, widespread pain and stiffness, tender points in deep muscles, and nonrestorative sleep. More than half of the chronic patients develop arthritis in the larger joints, especially the knees. Chronic arthritis cases suffer erosion of cartilage and bone (Gumstorp 1991, Schned and Williams 1992, Sigal 1990, Steere 1991). Long term deteriorating conditions include cardiac abnormalities (Reznick et al. 1986, Van Der Linde 1991) and neurologic complications (Halperin 1991, Kristoferitsch 1991, Shadick et al. 1994). Part of the neurologic complications arise from patient IgM antibodies cross-reacting with nerve cells. The flagellar protein (41 kDa) shares antigenic determinants with myelinated fibers and axons of the nervous system (Aberer et al. 1989, Sigal and Tatum 1988).

Extraordinary human aspects

A patient with cardiomyopathy for four years, was found to have antibodies to B. burgdorferi by ELISA and immunoblotting techniques which suggested chronic Lyme disease. B. burgdorferi was cultured from an endomyocardial tissue biopsy (Stanek et al. 1990).

Four cases of fetal borreliosis resulted in abortuses. Cultures of autopsy tissue were positive from all four liver samples and one heart sample (MacDonald 1986). Other cases of transplacental transmission and recovery of spirochetes from fetal tissues have been reported (MacDonald 1989, Schlesinger et al. 1985, Weber et al. 1988).

Certain major histocompatibilty complex (MHC) genotypes may be more susceptible to chronic arthritis and less responsive to antibiotic therapy but these conclusions are controversial (Steere et al. 1990, Herzer 1991, Pflueger et al. 1989, Ruberti et al. 1991).

Transmission of B. burgdorferi through blood products is possible but very unlikely except for endemic areas. Experimental inoculations into whole blood prior to processing and into processed blood components determined that B. burgdorferi was able to survive in blood products (Badon et al. 1989, Johnson, S. et al. 1990).

Antibacterial Therapy

Therapy with appropriate antibiotics instituted before damage due to sequelae occurs, usually results in full recovery but the disease relapses occasionally (Pfister et al. 1986, Weber et al. 1986). Antibiotics are only effective when the spirochetes grow and divide. Therapy must be aggresive when deep tissues are infected. Of 73 patients with Lyme arthritis, 70 (96%) had positive synovial fluid test results even after short term antibiotic therapy. Only seven of 19 (37%) had positive test results following prolonged antibiotic treatment (Nocton et al. 1994). B. burgdorferi may be capable of significantly reducing its growth rate, necessitating prolonged antibiotic treatment (MacDonald et al. 1990). The

spirochete was isolated from patients despite the conclusion of antibiotic treatment (Preac-Mursic et al. 1989, Schmidli et al. 1988). A uniform optimal antibiotic therapy has not been completely defined. One suggested protocol to treat early disease are doxycycline (or amoxicillin alternative) for adults and amoxicillin (or erythromycin), for children. Therapy should span a minimum of ten to twenty-one days but must be prolonged if symptoms persist (Rahn 1992).

Several studies measuring the minimum bacteriocidal concentration (MBC) or minimum inhibitory concentration (MIC) by various in vitro and in vivo methods were not always in general agreement on the effectiveness of certain antibiotics. Although erythromycin, tetracycline, and penicillin have been most commonly prescribed to treat patients with early Lyme disease, the three antibiotics are probably not the best choice for aggressive treatment. Ceftriaxone has a good record of patient recovery. Other antibiotics in approximate order of decreasing effectiveness were: doxycycline, cefuroxime, imipenem, augmentin, amoxicillin, ampicillin, lincomycin, mezlocillin, tetracycline, oxacillin, and ofloxacin. Chloramphenicol and penicillin G were less effective. Treatment with a single antibiotic may be less effective than a combination of antibiotics, especially when treating the various complications of advanced Lyme disease (Dattwyler et al. 1988, Johnson, R. 1989, Johnson R. et al. 1987 and 1990, Luft et al. 1989, Preac-Mursic et al. 1987, Philipson 1991). Long term treatment with ceftriaxone may cause biliary complications due to the sludging of precipitates of the antibody which obstructs or irritates the bile ducts through which it is excreted (CDC 1993, Shiffman et al. 1990).

Topical application of an antibiotic immediately following a tick bite, directly at the bite site, may be an effective treatment in the future. An experiment conducted on CD-1 mice found that topical application of either penicillin G, amoxicillin, ceftriaxone, or doxycycline prevented spirochetal infection after an infected tick fed to repletion. Erythromycin and tetracycline hydrochloride were less effective (Shih and Spielman 1993). Topical treatment may

become an effective prophylaxis following a tick bite or may supplement systemic treatment by diffusing antibiotics through the skin directly at the location of infection.

Comparisons of disease detection methods

Early detection is paramount to early treatment and complete patient recovery. Although polymerase chain reaction (PCR) methods of detecting B. burgdorferi are available, recovery of the spirochetes from specimens by in vitro culturing is the only unequivocal method of confirming the presence of Lyme disease (CDC 1992). A comparison of in vitro culturing and PCR amplification of a conserved *ospA* gene sequence found both methods were comparable in detecting natural infection in 108 live-trapped, wild P. leucopus mice. Consistent test results were obtained with 98 mice of which 73 were negative and 25 were positive. Six mice were PCR positive and culture negative. Four mice were culture positive and PCR negative. Additional test comparisons were conducted on 202 biopsies from various tissues collected from 39 of the mice. Of the 202 biopsies, 145 (79 organ and 66 ear samples) were negative by both methods, 29 (10 organ and 19 ear samples) were positive by both methods, 19 (16 organ and 3 ear samples) were culture positive only, and 9 (5 organ and 4 ear samples) were PCR positive only. Overall, culturing detected a greater number of naturally infected tissues than PCR with the exception of ear tissue. The results from the ear biopsies were more consistent between the two tests than any other tissue source (Hofmeister et al. 1992). Advances in PCR technique and the selection of more specific target B. burgdorferi DNA primer sequences have improved the sensitivity of detection by PCR amplification, challenging cultivation as the best method of clinical detection (Lebach et al. 1991, Moter et al. 1994). But PCR analysis requires equipment, reagents, and experienced technicians usually available only at well-developed scientific reference institutions. Recovery of the spirochetes by cultivation is dependent on the collection of an infected specimen, requires more time than other assays, and often yields contaminated cultures.

However, cultivation produces spirochetes that can be directly identified. A novel approach employed PCR amplification of in vitro cultures which eased technician visualization and may improve detection in poor culture conditions (Schwartz et al. 1993).

The difficulties encountered with serologic diagnosis of Lyme disease were addressed at the Second National Conference on Serologic Diagnosis of Lyme Disease held October 27-29, 1994. Public health officials grouped recommendations into four areas of concern: serologic test performance and interpretation, quality assurance practices, new test evaluation and clearance, and communication of developments in Lyme disease testing. The concensus approach to serologic detection of current or previous infection was to screen specimens using an enzyme immunoassay (EIA) or an immunofluorescent assay (IFA). Positive or equivocal specimens must then be tested using a standardized Western immunoblot assay. Positive Western immunoblot criteria are outlined below. When Lyme disease is suspected, a second serum specimen should be tested four to six weeks after the first analysis. An increase in antibody titer would support a diagnosis of Lyme disease. Patients with disseminated or late-stage Lyme disease exhibit a strong IgG response to several B. burgdorferi antigens (CDC 1995, Magnarelli 1995).

Serological testing is not only dependent on the amount of free circulating specific antibody produced by the immune response of the patient being tested, serologic testing also depends on the amount and type of spirochete antigen(s) used to bind the antibodies. Commercially available kits for the detection of antibodies against B. burgdorferi have varying sensitivities and specificities due to variability in the antigenic component (Callister et al. 1994, Mitchell et al. 1994, Schmitz et al. 1993). Test reagents vary between manufacturers. Variable performance was observed between different reagent lots from the same manufacturer (Mitchell et al. 1994). Laboratory proficiency testing demonstrated inconsistent inter- and intra-laboratory serologic results using immunofluorescent assays (IFA), enzyme immunoassays (EIA), and Western immunoblots (Callister et al. 1994).

Serologic testing was often negative early in the disease despite the presence of the hallmark erythema migrans (EM) rash (Magnarelli et al. 1987a, Rahn and Malawista 1991, Russell et al. 1984, Shrestha et al. 1985, Steere 1989). The lack of serologic detection early in the disease may be due to antibody sequestration in immune complexes (Schutzer et al. 1994). False seropositives arise from antibody cross-reactivity in patients with rheumatoid arthritis, rheumatic fever, systemic lupus, infectious mononucleosis, Rocky Mountain spotted fever, syphilis, and acute bacterial endocarditis (Callister et al. 1994, Cohen 1991,Kaell et al. 1990, Zoschke 1992). Responding to antibiotic therapy reduces the immune response and the ability to detect serum antibodies. Specific IgM antibody response may not develop until four to six weeks after infection but this is controversial (Schutzer et al. 1994, Zoschke 1992). During the first six weeks of infection, significant levels of specific IgG antibodies are present in only 50-60% of Lyme patients (Callister et al. 1994). Western immunoblot testing performed during the first four weeks of disease progression should assay for both IgG and IgM antibodies (CDC 1995). Previous to recent advances in serodiagnosis, a serosurvey of patients with varying stages of Lyme disease, found those with erythema migrans were only 20-50% seropositive, patients with neuroborreliosis were 70-90% seropositive, while arthritis cases were 90-100% seropositive (Wilske 1988). The enzyme immunoassay (EIA) is the preferred screening test for laboratories with EIA capabilities. EIA results are quantified by a spectrophotometer and less prone to error by human interpretation. Enzyme immunoassays were more sensitive and equal to or more specific than immunofluorescence testing (Cutler and Wright 1989, Lindenmayer et al. 1990). Western immunoblotting must be used to confirm EIA or IFA results (CDC 1995, Johnston et al. 1992). Due to the lack of standardized detection assays with high sensitivity and specificity, a diagnosis of Lyme disease should first rely on clinical observations and epidemiologic evidence (Magnarelli 1995).

Characterization of T-cell response

T-cell proliferation has been evaluated for its usefulness in diagnosing Lyme disease. This method was laborious and not specific. But the added information could be useful in attaining a well rounded diagnosis (Dressler et al. 1991, Zoschke et al. 1991). The T-cell response may be due to presentation of OspA or multiple antigenic epitopes (Lengl-JanBen et al. 1994, Roessner et al. 1994).

Histologic analysis

Histologic and microscopic identification of B. burgdorferi spirochetes in clinical specimens is extremely difficult due to the variable presence of spirochetes in tissue specimens. In nonspecific histologic staining methods, the spirochetes may be indistiguishable from muscle and connective tissue. The absence of distinct colonies precludes obvious visualization (Aberer and Duray 1991, Barbour 1989a, Duray 1989b, Zoschke 1992). An immunohistochemical staining method using polyspecific antibody to B. burgdorferi required laborious scanning to observe small clusters of three to four spirochetes sparsely distributed in tissue specimens from infected gerbils. This procedure produced very low sensitivity, detecting spirochetes in only 39% of known positive specimens (Lebech et al. 1995).

Immunofluorescent assay

Detection of patient antibody specific to B. burgdorferi using an indirect immunofluorescent assay (IFA) is not standardized and less objective than an EIA. IFA testing became less subjective as technicians gained experience interpretting results (Mitchell et al. 1994). Fluorescent reactions quickly dim. Acetone-fixation caused the spirochetes to swell and develop outer membrane blebs. The cytopathic effects were lessened with formalin or methanol-fixed preparations but the fluorescent intensity was reduced (Aberer and Duray 1991, Nadelman et al. 1990).

Enzyme immunoassay

An enzyme immunoassay (EIA) may be used to detect patient serum antibodies. An EIA is well suited for processing large numbers of specimens generated by a field surveillance study but early applications experienced limited usefulness in the serodiagnosis of Lyme disease due to low sensitivity (Ciesielski et al. 1989, Nadelman et al. 1990). To improve the reliability of EIA testing, various B. burgdorferi antigens have been tested. Use of purified flagellar antigen fraction improved the sensitivity of previous EIA methods for detecting IgM or IgG antibodies (Hansen and Åsbrink 1989, Hansen et al. 1991). B. burgdorferi whole cell sonicate based EIA testing yielded more true positive results than flagellar antigen (41kDa) based ELISA testing, even though a strong antibody response was directed against the flagellar antigen (Campbell et al. 1995). An extract of major proteins (MW = 34, 39, 59, and 68 kDa) had comparable sensitivity but greater specificity compared to a whole cell sonicate (Magnarelli et al. 1989). Recombinant OspC (21-22 kDa) provided a positive predictive value of 100% and a negative predictive value of 74% in a study of sera from 74 culture positive individuals and 75 negative controls (Padula et al. 1994).

Other methods of improving EIA testing have focused on the immune antibodies produced through the course of the disease, including differential testing for either IgG or IgM (Magnarelli and Anderson 1988a, Magnarelli et al. 1994). Sensitivity could be improved in borderline positive or suspect negative results by detecting antibodies that were sequestered in immune complexes. By precipitating and then dissociating the immune complexes, previously seronegative results became positive (Schutzer et al. 1990). Sera from syphillus patients cross-reacted with EIA testing (Magnarelli et al. 1994). An adsorptive procedure improved specificity by pretreating to remove cross-reactive antibodies (Fawcett et al. 1989).

Western immunoblot

Western immunoblotting also detects specific antibodies in the patient's serum directed against specific spirochetal antigens. The antigenic variability among various isolates of B. burgdorferi used to detect serum antibodies has reduced the effectiveness of immunologic methods, especially western immunoblotting. Antigen band profiles and band intensities vary greatly depending on spirochete strain and the number of in vitro passages prior to antigen seperation. Much effort has been put into standardizing results. Although certain specific immunoblot patterns indicate positive results, standardized criteria have not been determined (Dressler et al. 1993, Engstrom et al. 1995, Grodzicki and Steere 1988, Karlsson et al. 1989, Ma et al. 1992, Stiernstedt et al. 1991, Zoller et al. 1991). The Second National Conference on Serologic Diagnosis of Lyme Disease (October 27-29, 1994) recommended the criteria for a positive IgM immunoblot is the presence of at least two of the following three bands: 21 to 24 kDa (OspC), 39 kDa (BmpA), and 41 kDa(Fla). A positive IgG immunoblot consists of at least five of the following ten bands: 18 kDa, 21 to 24 kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (Fla), 45 kDa, 58 kDa (not GroEL), 66 kDa, and 93 kDa (CDC 1995, Dressler et al. 1993, Engstrom et al. 1995).

A survey of 578 human samples found serum antibodies recognized fourteen different protein bands (Ma et al. 1992). Human serum specimens taken early in the disease exhibited a strong antibody response to the flagellar (41-kDa) band (Hansen et al. 1988b, Grodzicki and Steere 1988, Wilske et al. 1986, Zoller et al. 1991). Purified 41-kDa antigen increased the effectiveness of immunologic testing (Hansen et al. 1988b, Magnarelli et al. 1989). Immunoblotting was more sensitive and yielded positive results earlier in the disease than any other assay for Lyme disease antibodies. False positives due to cross-reactivity have been observed (Coleman and Benach 1992, Grodzicki and Steere 1988, Guy and Turner 1989, Karlsson 1990, Magnarelli et al. 1987a). Immunoblot patterns cross-reacting with four B. burgdorferi strains

against sera from seropositive dogs produced heterogeneous patterns with five consistent intense bands (Greene et al. 1988). The use of Western immunoblots in the diagnosis of Lyme disease should be restricted to confirmation testing (Callister et al. 1994, Schmitz et al. 1993, Zoller et al. 1991).

Detection of specific DNA or RNA

PCR does not require viable spirochetes to be able to detect the presence of infection. However, specimen condition did affect PCR results in a comparison of known positive tissue tested fresh or formalin preserved and paraffin-embedded in preparation for immunohistologic analysis (Lebech et al. 1995). Presently, there is no standard PCR assay for routine diagnosis of Lyme disease (Callister et al. 1994, Stiernstedt et al. 1991). Test specificity is dependent on the specificity of the DNA primer sequence. B. burgdorferi DNA sequences that have been amplified include portions of the flagellar sequence (Johnson, B. et al. 1992, Lebech et al. 1995, Picken 1992, Wise and Weaver 1991) and the *ospA* gene (Debue et al. 1991, Guy and Stanek 1991, Hofmeister et al. 1992, Malloy et al. 1990, Moter et al. 1994, Nocton et al. 1994). But these PCR analyses had varying degrees of success in obtaining positive results from known positive patients. The OspA fragment has also been amplified to detect B. burgdorferi in rodent ear punch specimens (Barthold et al. 1991) and rodent organ tissue extracts (Hofmeister et al. 1992). PCR amplification has also detected *ospA* gene sequences in museum specimens. Dried rodent skins collected from 1870 to 1919 that were preserved by dusting yielded positive reactivity in two of 280 specimens tested with an OspA amplification technique (Marshall et al. 1994). I. scapularis ticks stored in alcohol in a museum for nearly fifty years also yielded positive PCR amplification of the *ospA* gene from midgut tissues (Persing et al. 1990a). Detection of infection by PCR amplification of B. burgdorferi chromosomal DNA was sensitive to less than five copies of the genome, even in the presence of 10^6-fold eukaryotic DNA (Goodman et al. 1991, Rosa et al. 1989). Cross-reactivity with other Borrelia species (Lebach et

al. 1991) and Leptospira interrogans (Kron et al. 1991) has been observed. Specificity can be improved by using a second primer (Lebach et al. 1991) or sequence specific oligonucleotide probes (Kron et al. 1991, Schwan et al. 1989). PCR analysis may also be performed on the medium supernatant from in vitro cultures to supplement microscopic inspection (Livesley et al. 1994, Schwartz et al. 1993).

Detection by in vitro culturing

Recovery of B. burgdorferi spirochetes from an infected specimen establishes a definitive Lyme disease diagnosis. Detection by culturing is the only unequivocal method of confirming Lyme disease (CDC 1992). Most researchers rely on culturing as the standard for establishing a positive disease condition by which to measure the performance of other diagnostic detection methods. Specified protocols for the production of culture medium, incubation conditions, and culture sampling must be followed to effectively recover and grow the troublesome spirochetes.

B. burgdorferi spirochetes have been cultured from skin punch biopsies taken from the margin of erythema migrans lesions (Berger et al. 1985, 1992, and 1995, Mitchell et al. 1993, Stobierski et al. 1994, Van Dam et al. 1993). Antibiotic treatment reduced recovery of spirochetes from skin specimens (Nadelman et al. 1993). Human EM skin biopsies cultured in a BSK II medium without antibiotics, rabbit serum, and gelatin, resulted in delayed growth. The cultures were incubated at 35°C for three weeks, then 24°C thereafter. Detection of the spirochetes by darkfield microscopy required a minimum of 2.5 months incubation but took as long as 10.5 months incubation (MacDonald et al. 1990). A dual needle lavage method used to recover B. burgdorferi from the skin of rabbits has also been used to recover the spirochetes from EM areas of human patients (Piesman et al. 1991, Wormser et al. 1992).

Although difficult to isolate from human specimens, B. burgdorferi has been cultured from the blood (Benach et al. 1983, Berger et al. 1994, Nadelman et al. 1990), cerebrospinal fluid (CSF;

Karlsson et al. 1990), and synovial fluid (Nocton et al. 1994, Schmidli et al. 1988, Snydman et al. 1986), of patients with Lyme disease. Spirochetemia in humans is brief, restricting recovery from blood. Recovery from blood was improved with heparin as the anticoagulant and when a larger volume (0.2 to 0.4 mL) of blood inoculum was cultivated. CSF specimens from patients with neuroborreliosis had a low isolation frequency. Centrifuging fluid specimens prior to cultivation helped improve sensitivity of recovery. For early detection of Lyme disease, culturing can be more sensitive than antibody detection assays. Of 86 culture positive patients with EM, only 40% had positive antibody titers. As the disease progressed, the ability to recover spirochetes decreased. Seropositive patients that received antibiotic therapy prior to specimen collection, significantly inhibited recovery of organisms (Stiernstedt et al. 1991).

Early development of borreliae culturing techniques

Early investigators studying human relapsing fever could only cultivate spirochetes in laboratory animals. In 1906, a spirillium (later classified as a Borrelia sp.) was cultured in dialysis sacks filled with sera implanted in the peritoneum of rats and rabbits (Novy and Knapp 1906). Their cultivation successes demonstrated that the spirochetes were not obligate intracellular parasites and could be passed indefinitely. However the concentration of spirochetes in the dialysis bag was never as high as the concentration of spirochetes in the blood of an infected animal. Another in vitro medium consisted of citrated human blood and rat blood incubated at room teperature (Norris, C. et al. 1906). Limited success was achieved in 1912 cultivating borreliae strains at 37°C in a paraffin sealed medium containing a few drops of blood and human or rabbit ascitic fluid that formed a loose fibrin matrix (Noguchi 1912). Although one Borrelia sp. isolate was passed 29 times, other isolates could not be maintained.

During the 1920's and 1930's, several media formulations supported the growth of various spirochetes with no loss of

virulence during subculturing. Some of the in vitro parameters held constant were: a serum constituent in the medium, a thickening agent (agar or fibrin from tissue), and maintaining the pH between 7.0 to 8.2 (7.2 to 7.4 was optimal). A medium containing horse or rabbit serum, saline, peptone broth, and rabbit blood, covered with mineral oil, was able to maintain borreliae cultures for three to seven weeks incubated at 28 to 30°C (Kligler and Robertson 1922). Another recipe was based on the combination of autoclaved cow or rabbit brains combined with animal serum (Aristowsky and Hoeltzer 1925, Eberson et al. 1931). A third recipe combined chicken egg, rabbit serum, and glucose (Bruynoghe 1928, Talice and Surraco 1929). These are three of the ingredients for the standard medium used today, substituting bovine albumin for the chicken egg. Borreliae were successfully cultivated in chicken embryos with unlimited passages (Anderson and Magnarelli 1992, Bohls et al. 1940, Oag 1939). Propagation in 7- to 12-day old fertilized eggs were still used as late as 1960 and successfully completed 35 passages over four months (Reiss-Gutfreund 1960).

Dr. Richard Kelly's discoveries

An adequate in vitro culture medium was not found until 1971 when Dr. Richard T. Kelly reported cultivation of B. hermsi (Kelly 1971). Many of the ingredients in his original formula are still used today. One key ingredient he added was N-acetylglucosamine, a component of chitin in tick cuticle (Hackman and Filshie 1982). He theorized that since this ingredient was present at high concentration in the tick it may enhance borreliae growth. Much later, researchers discovered that borreliae directly incorporated N-acetylglucosamine into peptidoglycan (Beck et al. 1990). By deleting one medium ingredient at a time, Dr. Kelly showed that the absence of certain ingredients like N-acetylglucosamine or albumin was detrimental to spirochete growth (Kelly 1971).

In 1976, Dr. Kelly evaluated the efficiency of various modifications to his original formula on the cultivation of five B. species but not B. burgdorferi. He devised three improved medium

variations to culture B. hispanica (Table 1), B. hermsii (Table 2), and B. recurrentis (Table 3). The media Dr. Kelly developed were not interchangeable. One medium formula could not support the growth of a variety of borreliae. After 150 passages some infectivity was lost (Kelly 1976).

Table 1. Dr. Kelly's medium for culturing B. hispanica

Solution 1:	Hanks' BSS 10X w/o $NaHCO_3$	
	w/ phenol red	13.50 mL
	Glucose	0.30 g
	Bovine albumin fraction V	3.00 g
	Sodium citrate-dihydrate	0.07 g
	Sodium pyruvate	0.08 g
	Proteose peptone #2 (Difco)	0.33 g
	Proteose peptone #4 (Difco)	0.30 g
	N-acetylglucosamine	0.04 g
	Choline chloride	0.01 g
	Glutamine	0.05 g
	QS w/ dd water to final vol.	95.00 mL
	pH adjusted to 7.8	
Solution 2:	Sodium bicarbonate, 2% dd H_2O solution	
	Prepared fresh each time	
Combine:	Solution 1	95.0 mL
	Solution 2	5.0 mL

Sterilize by filtration (0.22 μm). Dispense 5 mL into sterile, screw cap borosilicate tubes (13 X 100 mm) with Teflon liners. To each tube add, 0.5 mL of sterile rabbit serum, 0.1 mL of L-cystine solution (5.0 mg/L, pH 1.0, membrane sterilized), and 2.9 mL of sterile distilled water. Mix by inversion and adjust pH to 7.8 with 1N NaOH. Inoculate culture tubes with 0.1 mL of citrated plasma containing B. hispanica and incubate at 35°C.

Table 2. Dr. Kelly's medium for culturing B. hermsii

Solution 1:	Disodium phosphate ($7H_2O$)	26.52 g
	Monosodium phosphate (H_2O)	1.03 g
	Sodium chloride	1.20 g
	Potassium chloride	0.85 g
	Magnesium chloride ($6H_2O$)	0.68 g
	Glucose	12.75 g
	Proteose peptone #2 (Difco)	5.95 g
	Tryptone (Difco)	2.55 g
	Sodium pyruvate	1.06 g
	Sodium citrate ($2H_2O$)	0.47 g
	N-acetylglucosamine	0.53 g
	Double distilled water	1.00 L
	Stored at -20°C	

Solution 2: Bovine albumin, fraction V, 10% dd H_2O solution. Adjust pH to 7.8 with NaOH. Stored at -20°C.

Solution 3: Sodium bicarbonate, 4.5% dd H_2O solution. Prepared fresh each time.

Solution 4: Gelatin, 7% dd H_2O solution, autoclaved at 115°C for 15 minutes. Stored at 4°C.

Solution 5: Phenol red, 0.5% dd H_2O solution. Stored at 4°C.

Combine:	Solution 1	80.0 mL
	Solution 2	34.0 mL
	Solution 3	4.0 mL
	Solution 5	0.7 mL
	dd H_2O	1.3 mL

Filter sterilize (0.22 μm) under pressure. Dispense 6 mL into sterile, screw cap 9 mL borosilicate tubes (13 x 100 mm) with Teflon liners. Add 2 mL of liquified Solution 4 and 0.5 mL of sterile rabbit serum to each tube. Mix by inversion. Secure caps tightly and store completed tubes at room temperature. Use within 30 days.

Table 3. Dr. Kelly's medium for culturing B. recurrentis

Solution 1:	Disodium phosphate ($7H_2O$)	0.020 g
	Sodium chloride	1.380 g
	Potassium diphosphate	0.012 g
	Calcium chloride ($2H_2O$)	0.033 g
	Sodium citrate ($2H_2O$)	0.034 g
	Glucose	0.550 g
	Proteose peptone #2 (Difco)	1.300 g
	Choline chloride	0.003 g
	Asparagine	0.011 g
	Bovine albumin, fraction V	4.400 g
	Yeastolate (Difco)	0.264 g
	Double distilled water	100.000 mL
	Adjust pH to 7.8	
	Stored at -20°C	
Solution 2:	Sodium bicarbonate	1.40 g
	Ascorbic acid	0.11 g
	Double distilled water	100.00 mL
	Prepared fresh each time.	
Solution 3:	Gelatin, 10% dd H_2O solution.	
	Autoclaved at 115°C for 15 minutes.	
	Stored at 4°C.	

Combine 100 mL of Solution 1 and 10 mL of Solution 2. Mix and filter sterilize (0.22 μm). Dispense 4 mL into sterile, 9 mL screw capped culture tubes. Add 1.5 mL of liquified gelatin Solution 3, 0.5 mL sterile rabbit serum, and 2.5 mL sterile distilled water to each tube. Mix the 8.5 mL volume of medium by inversion and inoculate with 0.1 mL citrated plasma containing B. recurrentis. Incubate at 35°C.

Dr. Kelly's observations further characterized the metabolism of Borrelia species. Certain borreliae could now be passaged 100 times and still maintain infectivity for mice. Dr. Kelly made several key observations characterizing the growth kinetics of B. hermsi. He

observed the generation time was 18 hours. After seven days incubation, the cultures reached a maximum concentration of 3 to 5 x 10^7 spirochetes per milliliter. He first suggested the microaerophilic nature of the spirochete by observing that the culture grew poorly when exposed to aerobic conditions. He also tested the ability of the medium to support growth from different concentrations of inoculum. A culture could proliferate from a lower concentration when gelatin was added creating a semi-solid medium. The 15th and 26th subcultures were still infective when inoculated into mice. The ingredients in "Kelly's medium" constitute most of the formula for the medium currently preferred for culturing B. burgdorferi (Kelly 1976).

Dr. Herbert Stoenner's improvements

In an effort to study the biology of B. hermsi, Dr. Herbert G. Stoenner found that Kelly's medium required an inoculum of more than 800 spirochetes to establish a culture. Displeased with these observations, Dr. Stoenner set out to improve the results. Although the ingredients and stock solutions were the same as Kelly's medium, Dr. Stoenner modified the method of medium preparation. All components except the gelatin solution were combined, mixed, and filtered through an ultrafine, sintered-glass filter instead of a 0.22 μm membrane filter. After the autoclaved 7% gelatin solution was added, the complete medium was dispensed into the culture tubes. Dr. Stoenner also experimented with modifying the serum source. Guinea pig and sheep serum were unsuitable. Fetal calf serum generated active growth comparable to rabbit serum. Increased aggregate forms were present in the medium supplemented with the fetal calf serum (Stoenner 1974)

Dr. Stoenner later developed "fortified Kelly's medium" by adding yeastolate and a tissue culture medium, CMRL 1066 (Gibco laboratories, Grand Island, NY), containing several nutrients and growth factors. He found that CMRL medium gradually lost its nutritional potency as it neared the expiration date, about six months. A precipitate formed after adding these supplements so

coarse prefiltration was performed. B. hermsii cultures could now be grown from a single organism. Spirochetemic blood specimens were frozen with 10% glycerol at -68°C. The frozen stock remained viable. Antigenic variation resulted from spontaneous conversion at a rate estimated between 10^{-4} to 10^{-3} per cell per generation (Stoenner et al. 1982). Dr. Stoenner improved medium performance. He also simplified medium preparation which made fortified Kelly's medium more homogeneous and less likely to be contaminated.

Dr. Alan Barbour's modfications

Dr. Alan G. Barbour was the first to culture the newly recognized spirochete B. burgdorferi in 1983 (Barbour 1984). His culturing experiments were a continuation of the work of his mentor Dr. Stoenner. In vitro growth of B. burgdorferi was characterized. The optimal incubation temperature was 30 to 35°C. At 35°C, the population doubling time for B. burgdorferi was approximately 11 to 12 hours. Microaerophilic conditions were maintained by incubation in an increased carbon dioxide atmosphere or by almost completely filling the culture tube with medium to minimize the dissolved air content in the medium near the bottom of the culture tube. Dense spirochete growth was obtained from an inoculum containing as few as one to two spirochetes. At early stationary growth phase, the density of spirochetes was 0.5 to 1 X 10^8 spirochetes per mL (Barbour 1984, Barbour et al. 1983).

Dr. Barbour cultivated spirochetes from I. ricinus ticks (from Switzerland) in a medium formulation he termed Barbour-Stoenner-Kelly (BSK) medium. BSK medium did not contain Yeastolate but included glutamine in the CMRL 1066 solution (Barbour et al. 1983). Dr. Barbour prepared a variation of BSK medium, BSK I, solidified with 0.8% agarose in petri plates. The plated cultures incubated in a candle jar produced a lawn of growth. Dr. Barbour's BSK II medium included Yeastolate but deleted glutamine from the CMRL 1066 component. BSK II medium was used to culture B. burgdorferi spirochetes from Ixodes sp. tick midgut pools sent by Dr. Willy

Burgdorfer. BSK II was also used to grow some of the relapsing fever borreliae (Barbour 1984).

Some authors interchange BSK for BSK II when citing the medium formulation published in 1984. But the BSK formulation was not the same as the BSK II formulation. Furthermore, BSK II medium contained a combination of two antibiotics, nalidixic acid and 5-fluorouracil, to inhibit the growth of bacterial contaminants (Barbour 1984). BSK II medium is widely used by researchers culturing B. burgdorferi. The formulation for BSK II is in Table 4.

Table 4. Dr. Barbour's BSK II medium formulation

Combine in the following order:

Glass-distilled water	900.0	mL
CMRL 1066, w/o glutamine	100.0	mL
Neopeptone	5.0	g
Bovine albumin	50.0	g
TC Yeastolate	2.0	g
HEPES buffer	6.0	g
D-glucose	5.0	g
Sodium citrate	0.7	g
Sodium pyruvate	0.8	g
N-acetylglucosamine	0.4	g
Sodium bicarbonate	2.2	g

At 20 to 25°C, adjust the pH of the medium to 7.6 with 1 N NaOH. Add 200 mL of a 7% gelatin solution. Sterilize by filtration (0.22 μm) under air pressure. Store medium at 4°C until needed. Before aliquoting into culture tubes, add unheated, trace-hemolyzed, rabbit serum to a final concentration of 6%. Dispense into capped glass or polystyrene tubes or bottles. Fill containers to 90% full capacity. After inoculating, secure caps tightly and incubate at 34 to 37°C.

Over the next two years of cultivating the Lyme spirochetes, Dr. Barbour observed the effects of several variations of growth conditions. RPMI 1640 could be substituted for CMRL 1066. CMRL 1066 and neopeptone could be replaced by brain heart infusion (BHI) broth with 0.5% Casamino acids and salts solution. CMRL 1066 without glutamine was preferred. Poor growth was obtained with serum-free medium in the presence of high grade albumin. Scanning electron photomicrographs revealed outer membrane blebs. Initial isolations and early subcultures tended to aggregate into clumps of spirochetes. When separated by vortexing, the aggregates reassembled. Dr. Barbour suggested the stickiness may be important in binding to tick or host tissues. The formation of large aggregates may be a defense mechanism to avoid phagocytosis (Barbour 1986).

Utility of BSK II medium ingredients

In a synthetic media preparation, the concentration of each ingredient must closely approximate the microbe's natural environment. Medium ingredients derived from natural hosts provide a more natural spectrum of nutrients needed. The ingredients in BSK II medium that provide essential nutrients are: TC Yeastolate (vitamin B complex), bovine albumin (source of proteins), CMRL 1066 tissue culture medium (multi-nutritional), rabbit serum (multi-nutritional), neopeptone (partially hydrolyzed protein), and N-acetyl-D-glucosamine (a vital peptidoglycan component). The BSK II medium components added to enhance B. burgdorferi's energy cycle are: D-glucose (a carbon source), sodium pyruvate, and sodium citrate (intermediates). Ingredients without direct nutritional value but promote the spirochete's well-being are: gelatin (thickening agent), sodium bicarbonate (a physiological buffer), and HEPES (a medium buffer). Specific antibiotics are added to eliminate or inhibit the growth of a broad range of contaminants without inhibiting B. burgdorferi. Isolates have been stored frozen in 10 to 30% glycerol in BSK II medium and kept for several months (Anderson 1989c, Austin 1993). When compared to Amies broth, hypertonic Columbia broth, distilled water, physiologic saline,

phosphate buffered saline, and modified Stuart broth, BSK II medium was determined to be the best medium for transport of infected rodent tissues (Campbell et al. 1994).

Modifications to BSK II medium

Currently, BSK II medium and variants largely based on Kelly's formula are the only media available for the recovery of B. burgdorferi spirochetes from infected specimens. Despite this success, the spirochete undergoes transformations during in vitro culturing. Morphologic changes, loss of some plasmids, and antigenic alterations occur within a short period of time despite passage to fresh medium (Aberer and Duray 1991, Schwan and Burgdorfer 1987, Schwan et al. 1988a). Often the changes result in a loss of infectivity. Other disadvantages of the medium are that it is costly, difficult to prepare, and has a short shelf life. To improve in vitro cultivation, modifications have included varying medium preparation procedures, ingredients, medium consistency, culture vessels, and incubation atmosphere.

Some preparation protocols dissolve the peptone ingredient in solution prior to adding it (Anderson 1989c, Anderson and Magnarelli 1992, Coleman and Benach 1992). The order in which sterilized ingredients are added may also vary (Berger et al. 1992). CMRL 1066 with glutamine has been substituted for CMRL 1066 without glutamine. Furthermore the stored medium was supplemented with fresh 3% L-glutamine (Johnson, S. et al. 1984, Rawlings et al. 1987). L-Glutamine, a primer for the citric acid energy cycle, is hydrolyzed into glutamic acid and ammonia. A modified formula containing RPMI 1640 (Sigma Chemical Co., St. Louis, MO) substituted for CMRL 1066 resulted in poor growth (Austin 1993). To avoid the inclusion of phenol red present in CMRL 1066, a salts solution was substituted for CMRL 1066 in a modified medium formulation (BSK-A) which successfully supported growth (Williams and Austin 1992). Initial cultures of B. burgdorferi (strain Sh-2-82) in BSK-A had a slow generation time (28-32 hours) but after two or three passages the generation time improved to about 15 hours (Austin 1993). Isolates

grew equally well in media formulations with or without the addition of the reducing compounds DL-dithiothreitol, L-cysteine HCl, and superoxide dismutase (Anderson et al. 1986a, Anderson and Magnarelli 1992).

B. burgdorferi was successfully recovered from skin biopsies using a serum-free BSK II preparation combined with a cruder form of bovine (Berger et al. 1985). However, subsequent researchers employing the serum-free BSK medium formulation found the addition of rabbit serum improved growth (Åsbrink and Hovmark 1985, Austin 1993). Serum-free BSK medium is used for obtaining B. burgdorferi spirochetes for addition to or co-cultivation with tissue cells in culture (Backenson et al. 1995, Coleman et al. 1995). Fetal calf serum was substituted for rabbit serum; however, no explanation for the substitution and no effect on spirochete growth was given (Pachner et al. 1993). Albumin from various commercial sources and different lots from the same commercial source resulted in varied growth (Callister et al. 1990). In another medium preparation called MKP, the amount of bovine albumin was increased from 50 to 70 grams (Preac-Mursic et al. 1986).

A commercially produced modification (BSK-H medium, Sigma, #B-3528) is available for researchers unable to produce BSK II medium. BSK-H does not contain gelatin, agarose, or rabbit serum. A recommended rabbit serum supplement (Sigma, #R-7136) is also available through the same manufacturer. Other various components in BSK-H are in slightly different proportions than the formula for BSK II medium. Culturing experiments conducted in evacuated blood collection tubes and 96-well microtiter plates held in a candle jar, demonstrated the performance of BSK-H medium was similar to BSK II medium (Pollack et al. 1993). Although preliminary trials were stated to have evaluated over 30 batches of experimental medium preparations containing additions, deletions, or changes in the concentrations of ingredients, none of the ingredient variations or effects on the growth of B. burgdorferi were presented. The best experimental medium (BSK-H) was selected for further evaluation based on the following spirochete culture characteristics: frequent and active motility, straight coiling, uniform length, minimal

aggregation, rapid generation time, and abundant final density. Inoculation of media with decreasing concentrations of spirochetes produced positive growth in 10 of 12 culture tubes that received 10 spirochetes, 7 of 12 tubes inoculated with 5 spirochetes, and 5 of 12 cultures that received a single spirochete. The population doubling times were 10 to 11 hours at 37°C and 12 to 13 hours at 33°C. Infectivity was observed through 10 passages in vitro. After medium storage at 33 or 37°C for two months, the medium failed to support spirochete growth. However, freezer storage prior to adding the rabbit serum supplement was reported to maintain medium stability for up to eight months. The medium also served as a cryopreservative, maintaining viable spirochetes held up to eight months at -80°C. The availability of a standardized medium allows for direct comparisons of research results. However, the absence of a thickening agent and serum component is contrary to the composition recommended by the three researchers for which the standardized medium was named.

Motility is necessary for spirochetes to obtain more nutrients and move away from toxic waste products. Spirochetes prefer a viscous environment and would not survive in vitro when movement expends more energy than is gained as a result of the movement (Berg and Turner 1979). Spirochete growth was enhanced when gelatin was present in the medium (Kelly 1976, Barbour 1984). The motility of B. burgdorferi in various concentrations of hyaluronic acid fluids and methylcellulose standards diluted in BSK II and phosphate buffered saline (PBS) was measured to correlate velocity with viscosity (Kimsey and Spielman 1990). Low concentrations of agarose have been added to thicken the liquid BSK II medium (Anderson 1989c, Anderson et al. 1986a).

Higher concentrations of agarose produced solid BSK medium. Dr. Barbour's BSK I solid medium contained 0.8% agarose which supported a lawn of B. burgdorferi growth from an inoculum of about 10^8 spirochetes per milliliter (Barbour 1984). Increased concentrations of thickening agents and decreased concentrations of spirochete inocula deterred confluent growth and yielded translucent but distinct colonies of B. burgdorferi (Dever et al.

1992, Kurtti et al. 1987, Preac-Mursic et al. 1991, Williams and Austin 1992). Growth was obtained from surface inoculations after two to four weeks (Kurtti et al. 1987) although better plating efficiency was obtained by inoculating molten (37°C) agarose-BSK medium with spirochetes and pouring the mixture over solidified layer of agarose-BSK (Dever et al. 1992, Rosa et al. 1992, Sadzienne et al. 1992, Williams and Austin 1992). To efficiently culture B. burgdorferi on solid medium, the atmosphere of incubation must have reduced oxygen and slightly increased carbon dioxide produced by the use of a candle jar or within anaerobic conditions (Austin 1993). Distinct colony formations were useful for selecting clones, isolating the spirochetes from bacterial contaminants, and performing spirochetal susceptibility testing.

Dr. Preac-Mursic and colleagues (1991) performed an extensive study on the ability of six solid media, including BSK II, containing 1.5% agar to grow B. burgdorferi. Ingredient variations included sodium thioglycolate, equine serum, human plasma, Bacto brain heart infusion (Difco Laboratories, Detroit, MI), Bacto liver (Difco), and Minimum Essential Medium (MEM) Alpha medium (Gibco/BRL, Germany). Ten B. burgdorferi isolates from various sources were grown initially in liquid BSK II medium. For each isolate, four replicate plates were inoculated with either 0.01 mL or 0.05 mL of spirochetes ranging in concentration from 10^3 to 10^5 per mL. To reduce the oxygen concentration, the plates were incubated in three atmosphere conditions: a candle jar, with an anaerobic catalyst, or a catalyst suitable for the growth of Campylobacter species.

Of the six media formulations tested, PMR medium had the highest recovery and most diverse and best colony appearance. The formula for PMR medium was similar to BSK II except for the addition of sodium thioglycolate, equine serum, and human plasma and the deletion of sodium bicarbonate, rabbit serum, and bovine albumin. Solid BSK II medium supported growth but the colonies were not as diverse or well-defined. Growth was also obtained on solid MKP medium. The formulation for MKP medium (Preac-Mursic et al. 1986) was very similar to BSK II but with changes in the amounts of all ingredients and deletion of Yeastolate (Difco). Poor

growth was observed on BHIAM medium which was the same as PMR medium except for the addition of Bacto Brain Heart Infusion (Difco). Poor growth was also observed on TAROM medium which consisted of CMRL 1066 (Gibco), water, equine serum, human plasma, and Bacto Liver (Difco). No growth was observed on the MEM medium which contained MEM (Gibco), equine serum, and human plasma. Spirochete growth was more rapid and diverse when the culture plates were incubated anaerobically rather than within a candle jar (Preac-Mursic et al. 1991).

B. burgdorferi was successfully cultivated in multi-well microtiter plates filled with BSK II medium and incubated in a carbon dioxide enhanced atmosphere (Keller et al. 1994, Pollack et al. 1993, Rosa et al. 1992, Sadzienne et al. 1992, Sinsky and Piesman 1989, Walker et al. 1994). Some medium evaporation occurred over time, so the wells were occasionally topped off. Incubation of a low passage B. burgdorferi (strain Sh-2-82) in tubes of BSK II medium in ambient atmosphere (20% O_2 - 0.03% CO_2) lost infectivity after 15 to 17 passages while cultures held in a gas mix atmosphere (4% O_2 - 5% CO_2 - 91% N_2) maintained infectivity through more than 20 passages. The increased carbon dioxide concentration did not increase the acidity of the medium (Austin 1993). Filtration has been applied to mixed cultures to isolate spirochetes which are capable of migrating through the filter membrane (Johnson, R. 1981). Syringe tip filters (0.2 and 0.45 μm) were used to separate B. burgdorferi from experimentally contaminated cultures. The filtered culture was inoculated directly into a new tube of BSK II medium (Jobe et al. 1993). Researchers have included various combinations of antibiotics to inhibit the growth of contaminants.

Antibiotics used to confer BSK II medium selectivity

Different combinations of antibiotics have been added to selectively eliminate contaminates but allow the growth of B. burgdorferi. Antibiotics that B. burgdorferi has demonstrated resistance to and are added to confer medium selectivity are: amikacin, co-trimoxazole, 5-fluorouracil, gentamicin, kanamycin,

nalidixic acid, phosphomycin, rifampin, sulfonamides, and the antifungal, amphotericin B. Aminoglycocides such as amikacin (a kanamycin derivative), gentamicin, and kanamycin inhibit bacterial protein synthesis by binding at several ribosomal sites causing mistranslations of mRNA (Gale et al. 1981a). Co-trimoxazole (or trimethoprim-sulfamethoxazole) a sulfonamide, blocks bacterial folic acid metabolism by inhibiting dihydrofolate reductase and tetra-hydropteroic acid synthetase (Gale et al. 1981b). 5-fluorouracil, bacteriostatic for many organisms, inhibits the formation of thymine nucleotides by blocking the thymidylate synthetase reaction (Gale et al. 1981c). Nalidixic acid also inhibits DNA synthesis (probably by inhibiting DNA gyrase), may further degrade DNA, and has some lesser effects on RNA and protein synthesis (Gale et al. 1981c). Phosphomycin (fosfomycin or phosphonomycin) blocks cell wall synthesis by inhibiting amino acid incorporation into peptidoglycan (Gale et al. 1981d). Rifampin interferes with RNA synthesis by binding to the RNA polymerase beta subunit (Gale et al. 1981c). Amphotericin B complexes with sterols present in the fungal cell membranes which alters membrane permeability allowing detrimental leakage of small molecules such as ions, sugars, and amino acids (Gale et al. 1981b).

Some media preparations have used a single antibiotic (Anderson 1989a, Nelson et al. 1991, Preac-Mursic et al. 1986) or none at all (Kurtti et al. 1987, MacDonald et al. 1990, Williams and Austin 1992). Better recovery of spirochetes from duplicate mouse ear punch samples was obtained in BSK II without antibiotics than in BSK II with antibiotics (Barthold et al. 1991). Some culture protocols use a medium containing antibiotics for initial specimen incubation then transfer the specimen or positive spirochete growth to culture medium without antibiotics (Berger et al. 1992, Van Dam et al. 1993). A combination of antibiotics would collectively control a broader spectrum of contaminants. Combinations of antibiotics have included: nalidixic acid and 5-fluorouracil (Barbour 1984, Nadelman et al. 1990); kanamycin and 5-fluorouracil (Johnson, R. et al. 1984a); rifampin and phosphomicin (Schwan et al. 1988b); rifampin, phosphomycin, and amphotericin B (Hofmeister and Childs

1995, Schwan et al. 1993, Sinsky and Piesman 1989, Walker et al. 1994); gentamicin, sulfamethoxazole, and trimethoprim (Van Dam et al. 1993); phosphomycin, 5-fluorouracil, trimethoprim, and sulfamethoxazole (Morshed et al. 1993); and actinomycin D, nalidixic acid, gentamicin, chloramphenicol, neomycin, puromycin, and rifampin (Kurtti et al. 1993). In general, all B. burgdorferi cultures started from tissue samples have some contamination that necessitates the use of a selective medium. Examples of antibiotic combinations are in Table 5.

Table 5. Antibiotics used to confer BSK II medium selectivity

	Antibiotic agents used to confer selectivity of BSK medium (concentration in μg per mL)										
References	Amphotericin B	Ciprofloxacin	Cyclohexamide	5-fluorouracil	Gentamycin	Kanamycin	Nalidixic acid	Phosphomycin	Rifampin	Sulfamethoxazole	Trimethoprim
Anderson 1984	-	-	-	-	-	-	-	-	50	-	-
Barbour 1984a	-	-	-	200	-	-	100	-	-	-	-
Berger 1992	-	0.4	-	-	-	-	-	-	40	-	-
Ewing 1994	-	-	-	-	-	8	-	-	50	-	-
Hofmeister 1995	4	-	-	-	-	-	-	1000	10	-	-
Johnson 1984a	-	-	-	230	-	8	-	-	-	-	-
Livesley 1994	-	-	-	-	-	-	-	50	50	-	-
Morshed 1993	-	-	-	100	-	-	-	400	-	50	10
Nadelman 1990	-	-	-	100	-	-	100	-	-	-	-
Roehrig 1992	-	-	20	-	-	-	-	-	-	-	-
Schwan 1988b	-	-	-	-	-	-	-	100	50	50	10
Schwan 1993	10	-	-	-	-	-	-	100	50	-	-
Sinsky 1989	2.5	-	-	-	-	-	-	20	50	-	-
van Dam 1993	35.7	-	-	-	5	-	-	-	-	250	71.4

Evaluating in vitro growth of B. burgdorferi

Quantitation of spirochete concentration is almost universally performed with either a Petroff-Hausser cell counting chamber or a Neubauer cell counting chamber. Both chambers are exactly alike

except for the depth of the chamber which affects the volume of fluid being measured. Cell counting chambers were used by Kelly, Stoenner, Barbour, and subsequent researchers interested in quantifying spirochete generation times. A different method determined spirochete concentrations by first performing a serial ten-fold dilution to 10^{-3}, dispensing 6 μL of the suspension onto a slide (coverslipped), and then counting all the cells present on the entire slide preparation (Berger et al. 1985). Monitoring incorporation of tritiated adenine (^{3}H-adenine) into B. burgdorferi cells cultured in BSK II medium was used to measure growth in relation to the effectiveness of certain antibiotics (Pavia and Bittker 1988).

In liquid BSK II medium, spirochete isolates form various aberrant morphologies and configurations. The apical poles of an individual cell may become attached to form a ring. Cell division may be incomplete leaving two cells attached end to end. Membrane duplication appeared as thickening of an individual cell. Various sizes of cell aggregates or clumps were observed in low passage isolates. The configuration of the aggregates varied from being spherical to web-like. Clumping had an affect on concentration determinations. Lab adapted strains lost the propensity to form aggregate clumps (Aberer and Duray 1991, Barbour 1984, Berger et al. 1985, Ewing et al. 1994, Johnson, R. et al. 1990, Nadelman et al. 1990, Preac-Mursic et al. 1991). No correlation has been made between clumping and infectivity. The density of spirochetes in the aggregates have not been determined. On solid BSK II medium, two different B. burgdorferi isolates formed distinctly different colony forms (Kurtti et al. 1987). But colony morphology on solid agar media may not be a stable phenotype (Williams and Austin 1992).

Spirochete length, number of coils, amplitude of coils, and other ultrastructural variations were measured by electron microscopy. Spirochete length increased as the primary culture aged. The cells also tended to become less coiled after continuous culturing. Blebs in the outer membrane have appeared (Barbour and Hayes 1986, Garon et al. 1989, Kurtti et al. 1988). The blebs may be the result of pH alterations.

In vitro cultivation has resulted in the loss of plasmids (Barbour 1988b, Hyde and Johnson 1986, Schwan et al. 1988a), and changes in specific proteins (Bisset and Hill 1987, Schwan and Burgdorfer 1987, Wilske et al. 1986). After 11-15 serial passages performed over 6-8 weeks, expression of the OspB protein (34 kDa) was progressively weaker or lost entirely (Lane and Lavoie 1988, Schwan and Burgdorfer 1987, Schwan et al. 1988a, Wilske et al. 1988). Higher passage (>25) strains developed phenotypic stability as they adapted to in vitro culturing (Adam et al. 1991). B. burgdorferi isolated from human CSF lost a major protein (23 kDa) after subculturing for four months (Karlsson et al. 1990). The opposite effect occurred when an OspA protein (32 kDa) became more dominant after repeat culturing (Wilske et al. 1986). The protein changes result in antigenic changes (Schwan and Simpson 1991). Varying the albumin component of BSK II medium caused morphological cell changes and decreased the quality of an indirect fluorescent-antibody test (Callister et al. 1990).

Loss of infectivity in white-footed mice coincided with the loss of a 7.6 kb circular plasmid, a 22 kb linear plasmid and changes in protein expression and the LPS-like material (Schwan et al. 1988a). After 10 to 20 passages in culture, infectivity was either lost or dramatically decreased (Barbour and Hayes 1986, Moody et al. 1990, Schwan et al. 1988a and 1988b, Stanek et al. 1985). The age and genotype of laboratory mice affected the severity of Lyme borreliosis. Young laboratory animals with immature immune systems were more susceptible to rapid disease development (Barthold 1991, Barthold et al. 1990, Moody et al. 1990). Infectivity was also affected by the route of inoculation. The intradermal route produced the highest incidence of infection (Barthold 1991). Most blood and spleen specimens were culture positive in three days. At 10 days, all blood and spleen specimens were positive (Barthold et al. 1991). In addition to infectivity, expression of outer surface proteins and the subsequent immune response by the new host was also affected by the route of inoculation. Most notable was the absence of antibodies directed against OspA in rodents naturally infected by ticks compared with needle inoculated rodents that

produce an early strong immune response against OspA (Golde et al. 1994, Pachner and Delaney 1993, Roehrig et al. 1992).

Cocultivation with other cell lines

Cocultivation of B. burgdorferi and human endothelial cells (Coleman et al. 1995, Ma et al. 1991, Szczepanski et al. 1990) or neural cells (Garci-Monco et al. 1989, Thomas et al. 1994) have been used to study attachment and interaction. Low passage isolates of B. burgdorferi adhered better than isolates passaged in continuous culture. Adherence was inhibited by addition of monoclonal antibodies to B. burgdorferi antigens (Comstock et al. 1993). Spirochete adherence was also inhibited by antibodies to fibronectin and other subendothelial matrix components. Interaction of the spirochetes with cultured human umbilical vein endothelial cells demonstrated both intercellular and intracellular presence. Cocultivation of the spirochetes with either human fibroblast cells, Vero cells, mouse keratinocytes, or HEp-2 cells conferred protection from ceftriaxone antibiotic (Georgilis et al. 1992). Cocultivation with joint tissue from newborn LEW/N rats preserved spirochete arthritogenicity to newborn rats through 22 passages compared to a loss of arthritogenicity after 7 passages in BSK II (Guner 1994).

B. burgdorferi was cocultivated with embryonic tick cell lines from five different species including D. variabilis but not an I. species cell line. The tick cell cultures supported B. burgdorferi growth but at a much slower rate compared to BSK II cultures. Spirochetes passaged within tick cell cultures had longer generation times (27.1 ± 4.5 hours) than spirochetes in BSK II cultures (11.7 ± 2.2 hours). The spirochetes attached to the cultivated tick cells as soon as they came in contact with the cells. Spirochete growth declined significantly when fetal bovine serum (FBS) was replaced by rabbit serum in the L-15B medium. BSK II medium did not support tick cell lines. After 15 passages in BSK II medium, B. burgdorferi lost the ability to infect hamsters. Spirochetes cocultivated with tick cells retained infectivity after 50 passages over one year continuous cocultivation (Kurtti et al. 1988 and 1993).

MATERIALS AND METHODS

Medium preparation

Basal medium. The basal medium was prepared in glassware that was washed and rinsed with distilled water. One liter of base was prepared by combining the ingredients listed in Table 6.

Table 6. Basal medium preparation

To prepare one liter of medium base combine:		
Endotoxin-free purified water	900.0	mL
CMRL 1066, 10X w/o glutamine (Gibco BRL, #330-1540AG)	100.0	mL
Neopeptone (Difco, #0119-01)	5.0	g
TC Yeastolate (Difco, #5577-15-5)	2.0	g
HEPES buffer (Sigma, #H-0891)	6.0	g
N-acetylglucosamine (Sigma, #A-3286)	0.4	g
D-glucose	5.0	g
Sodium citrate	0.7	g
Sodium pyruvate	0.8	g
Sodium bicarbonate	2.2	g

Components were mixed using a magnetic stir bar. The mixture was poured into a pressure vessel and filter sterilized through a 0.2 μm filter (Gelman mini-capsule, #12122, Ann Arbor, Mi.) in a laminar flow biological safety cabinet. The basal medium was asceptically dispensed into sterile polystyrene tissue culture flasks with screw-tops. The volume of basal medium in each flask varied from 500 mL to 200 mL depending on plans for the production of each type of complete medium. The most efficient volume to aliquot to storage was 200 mL. Three mL aliquots of basal medium were aseptically dispensed into five mL (12 x 75 mm) sterile, snap cap polystyrene tubes to perform basal medium quality control by

incubating at 37°C for one week to check for contamination, turbidity, or pH change. The larger volumes were capped and stored at 2 to 8°C until needed for preparation of the final medium products. All medium variations contained the basal preparation.

Final medium products. Before the final ingredients were added, an aliquot of basal medium was removed from refrigerated storage and brought to room temperature. The ingredient amounts listed in Table 7 were combined to prepare each final product. After adding the antibiotics, three mL aliquots of final medium product were aseptically dispensed into five mL (12 X 75 mm) sterile, snap cap polysytrene tubes (Falcon #2054, Becton Dickinson, Lincoln Park, NJ).

Table 7. Final medium product

Ingredient volumes to prepare 258.5 mL final product:	
Basal medium aliquot	200.0 mL
Albumin (bovine or human sources)	10.0 g
Gelatin, 7% w/v solution (Difco, #0143-01)	40.0 mL
Serum (rabbit, human, or fetal bovine sources)	14.4 mL
Phosphomycin, 100 mg/L final concen. (Sigma, #P-5396)	2.0 mL
Rifampin, 50 mg/L final concen. (Sigma, #R-7382)	2.0 mL
Amphotericin B, 2.5 mg/L final concen. (Squibb, #NDC 003-0437-30)	0.1 mL

Human albumin, human serum, and fetal bovine serum substitutions were made for the bovine albumin and rabbit serum components. Individual component substitutions and combinations of substitutions were tested. The BSK II medium modifications (and designations) consisted of the following: human serum (BSK II-S) or fetal bovine serum (BSK II-F) substituted for rabbit serum, human albumin (BSK II-H) substituted for bovine albumin, bovine albumin and human albumin (BSK II-H50) added in equal quantities, both human albumin and human serum (BSK II-HS) substitutions, and both human albumin and fetal bovine serum (BSK II-HF) substitutions. Second batches of media supporting the fastest generation times

were prepared for three follow-up experiments: to repeat comparisons of the generation times of cultures started from frozen stock, for comparisons of generation times among active passages, and for growth comparisons from low spirochete concentration inocula. Table 8 lists the six different combinations of ingredient substitutions prepared in addition to the standard Barbour-Stoenner-Kelly II (BSK II) medium.

Table 8. Combinations of ingredient substitutions

	Albumin source	Serum source	Nomenclature
1.)	Bovine	Rabbit	BSK II
2.)	Bovine	Human	BSK II-S
3.)	Bovine	Fetal bovine	BSK II-F
4.)	Human	Rabbit	BSK II-H
5.)	Bovine/Human	Rabbit	BSK II-H50
6.)	Human	Human	BSK II-HS
7.)	Human	Fetal bovine	BSK II-HF

Albumin components. All albumin components were stored at 2 to 8°C until needed. For each medium prepared, 200 mL of basal medium was poured into a sterile 1 L glass beaker. The albumin component (10 g per 200 mL) was slowly added to the base while mixing with a magnetic stir bar and glass rod. The suspension was thoroughly mixed until all the albumin dissolved into solution. Bovine albumin (ICN Biochemicals, #02-16006980, Cleveland, Oh.) was used to begin preparation of the standard BSK II medium and the medium modifications designated BSK II-S and BSK II-F. Human albumin (J. Eckenrode, Blood Derivatives Section, MDPH) was substituted for bovine albumin to begin preparation of the medium modifications designated BSK II-H, BSK II-HS, and BSK II-HF. Another modified medium preparation made with a combination of both bovine and human albumin components, each contributing 50% of the required amount of albumin, was designated BSK II-H50.

After the albumin dissolved, the pH was checked and recorded as the base pH. Then the pH was adjusted to 7.6 ± 0.2 by gradually

adding 1N NaOH while constantly stirring. The pH was recorded as the final pH. A 7% (w/v) gelatin solution was prepared in endotoxin-free water by slightly heating to dissolve the gelatin. After briefly cooling the gelatin solution in a 60°C water bath, 40 mL was added to the 200 mL of albumin-basal medium mixture and briefly swirled. The mixture was transported to a biological safety cabinet, poured into a pressure vessel, and filter sterilized (0.2 μm, Gelman mini-capsule) under pressure, into a sterile Ehrlenmeyer flask. The flask was immediately plugged with sterile gauze.

Serum components. Aseptic techniques were used to add 14.4 mL of filter sterilized serum to the mixture. The mixture was swirled to blend. Rabbit serum (prefiltered 0.22 μm, Pel-Freeze, #31126-1, Rogers, Ar.) was used to prepare the standard BSK II medium, BSK II-H, and BSK II-H50. Serum substitutions were sterilized by membrane filtration (0.22 μm, Millex-GS #SLGS0250S, Millipore Products Division, Bedford, Ma.) into sterile collection flasks. Human serum used to produce the first batch of BSK II-S and BSK II-HS came from pooled "clot tube" samples (American Red Cross, Lansing, Mi.). The one week old human sera samples had been subjected to routine blood banking analysis and storage. These BSK II-S and BSK II-HS batches were used for initial culture trials and growth comparisons from frozen stock. Human serum used to prepare BSK II-S and BSK II-HS for the active passage comparisons and the recovery experiment was harvested from a single donor (T. Smith). Fetal bovine serum (#14-501F, Whitaker Bioproducts, Walkersville, MD) was used to prepare BSK II-F and BSK II-HF.

Medium selectivity. Three agents were aseptically added to confer selectivity of the final medium product for B. burgdorferi: phosphomycin (Sigma Chemical Co., #P-5396, St. Louis, Mo.), rifampin (Sigma Chemical Co., #R-7382, St. Louis, Mo.), and amphotericin B (Fungizone, Squibb Pharmaceuticals, #NDC 0003-0437-30, Princeton, N. J.). All three agents were prepared according to the guidelines in the National Committee for Clinical Laboratory

Standards (1990). The final concentrations were: rifampin 50 mg/L, phosphomycin 100 mg/L, and amphotericin B 2.5 mg/L.

After the antibiotic and antifungal agents were added, the final medium product was thoroughly swirled to mix. Each product was aseptically dispensed in 3 mL aliquots into sterile, labeled, capped polystyrene 12 x 75 mm test tubes. A random sample of 10 tubes were selected for quality control and the rest were held at 2 to 8°C for at least one week while quality control was conducted.

Medium quality control. Quality control (QC) consisted of five checks, four were performed by the Michigan Department of Public Health (MDPH) Quality Control Unit. The medium must have no evidence of contaminant growth when held for one week within three conditional incubations: (1) at 35°C as dispensed in original culture tubes, (2) aliquoted into thioglycolate broth at 35°C , and (3) aliquoted into soybean, casein, digest broth at 23°C. After one week, the QC pH of the culture tubes held at 35°C must be 7.6 ± 0.2. The fifth check tested the ability of the medium to support the growth of known B. burgdorferi spirochetes. Periodically, additional tubes were selected at random, incubated uninoculated, and observed for contamination.

Sources of B. burgdorferi spirochetes

A field study collected duplicate ear punch biopsy specimens from white-footed mice (P. leucopus), deer mice (P. maniculatus), and eastern chipmunks (T. striatus) that were live-trapped in Menominee County, Michigan during the summer of 1990. One ear biopsy specimen per rodent was cultured in BSK II medium. The spirochetes recovered were identified as B. burgdorferi by an immunofluorescent assay (Dr. Babara Robinson-Dunn, Michigan Department of Public Health, unpublished data) and polymerase chain reaction analysis (Dr. Michael Kron, Michigan State University, unpublished data). The duplicate ear biopsy specimens were frozen at -70°C in 30% glycerol-saline solution and stored in the MDPH culture collection for 8 to 18 months prior to use in this study.

Seven of the duplicae ear biopsy specimens provided B. burgdorferi isolates used in this study. The isolates were designated according to collection data: county, site, and rodent number (Table 9).

Table 9. Rodent sources of Borrelia burgdorferi spirochetes

	Rodent type	Isolate designation
1.	White-footed mouse (P. leucopus)	MEN-H1
2.	White-footed mouse (P. leucopus)	MEN-M3
3.	White-footed mouse (P. leucopus)	MEN-TC1
4.	Eastern chipmunk (T. striatus)	MEN-TC2
5.	White-footed mouse (P. leucopus)	MEN-W3
6.	Deer mouse (P. maniculitis)	MEN-W5
7.	White-footed mouse (P. leucopus)	MEN-W6

All biopsy and culture manipulations were performed in a laminar flow biological safety cabinet. The frozen ear punch biopsy suspensions were thawed at room temperature. Forceps were sterilized by dipping in ethanol and briefly flaming, then allowed to cool. The sterilized forceps were used to transfer the biopsy specimen into a culture tube containing medium. Specimens were cultured in each of the seven medium variations to provide spirochetes that evolved uniquely under each of the different nutritional conditions. Seven tubes of BSK II medium and seven tubes of BSK II-H medium were inoculated with 1 mL aliquots of thawed glycerol-saline solution from each of the seven ear punch biopsy storage vials to check for the presence of viable spirochetes suspended in the storage solution. All cultures were incubated at 31 ± 2°C. Up to 48 hours after the ear biopsy specimens were placed into culture tubes, small air bubbles formed on the hairy surfaces of the specimens which caused them to rise to the top of the medium. As necessary, the culture tubes containing the floating ear specimens were struck sharply to dislodge the air bubbles causing the specimens to sink to the bottom of the tube where microaerophilic conditions support B. burgdorferi growth.

The ear punch biopsy cultures were checked weekly for spirochete growth by using an autoclaved, disposable glass pipet to remove an aliquot of the culture medium near the specimen. Heavily contaminated cultures were not used for continuing analysis. The concentration of spirochetes per milliliter of medium harvested from biopsy cultures was determined with a Neubauer cell counting chamber (see spirochete concentration measurement). To prepare stock collections for experimental use and preserve cultivated spirochetes, one milliliter aliquots of B. burgdorferi cultures were frozen directly in the culture medium at -70°C without the addition of glycerol.

Sampling B. burgdorferi cultures

Prior to sampling, each culture tube was gently mixed by hand vortexing to distribute the spirochetes evenly throughout the three milliliter volume of medium. A small aliquot of the broth was withdrawn near the bottom the culture tube with an autoclaved, disposable glass or sterile plastic pipet. This was dispensed into two chambers of a coverslipped, Neubauer cell counting slide for examination and enumeration by phase-contrast microscopy under 400X magnification. Any excess culture remaining in the pipet was returned to the culture tube. To inhibit the Neubauer chamber preparations from drying out while awaiting enumeration, the preparations were placed on a rack above moist paper towels inside of a covered, shallow plastic transport box. The concentration of spirochetes was determined within each chamber. After each use every Neubauer slide was submerged in a 70% ethanol bath and the coverslip dislodged from the top. Excess culture remaining on the slide was scraped off with a wood dowel. After a minimum of 15 minutes in the ethanol bath, the slides were removed, meticulously cleaned with Kimwipes, and air-dried prior to using them again. To measure error between the counting chambers, all eight slides were prepared from the same tube culture and the spirochetes in each chamber were counted.

Culture tubes were evaluated to determine if gently vortexing by hand had a detrimental effect on growth. Six culture tubes were inoculated and undisturbed for four days. Six culture tubes were inoculated in parallel and vortexed every 24 hours for concentration enumeration. Five additional culture tubes were inoculated and subsequently sampled from both the bottom, middle, and the top of a hand vortexed culture to determine if the concentration of spirochetes was consistent throughout the tube.

B. burgdorferi concentration measurement

Counting spirochetes commenced at the surface of a 0.04 mm^2 grid square etched into the bottom of the counting chamber. The top of the chamber was determined by observing the point where the bottom of the coverslip started. The coverslip bottom was visualized by observing where several microscopic cells or other debris went out of focus and none came in to focus. To fully count all motile spirochetes suspended in one small cube (0.004 mm^3) of the chamber, counting commenced at the grid surface on the bottom of the chamber and continued while focusing up to the top of the chamber preparation located at the bottom of the coverslip. Individual spirochetes suspended at different levels within the counting chamber were readily distinguished by passing in and out of focus. The total number of spirochetes were counted within five cubes (0.02 mm^3). Only motile spirochetes were counted. The observation time was recorded to the nearest 0.5 hr. Cell morphologies, aggregates, nonmotile cells, and contaminants were qualitatively described.

Each counting chamber was filled with culture suspension by capillary action. A volumetric transfer pipet, capable of delivering a controlled volume of broth from near the bottom of the culture tubes was not available. The depth of the filled chamber preparations varied from the designed 0.1 mm depth. Therefore, the volume of culture suspension varied which affected spirochete enumeration. To correct for the volume variability, the raw number of spirochetes counted per chamber was adjusted proportionally

(Figure 1). The depth of every chamber-suspension preparation was measured at the four corner squares and at the middle square of the counting grid. This was accomplished by first focusing on the etched square surface of the chamber bottom and determining the number of focal units (numerically designated on the fine focus knob) needed to reach the top of the chamber. The average chamber depth measured at the five squares was calculated and recorded for every preparation. The proportion was based on a median depth measurement for all counting chambers of 70 focal units measured from the etched grid, bottom surface of the chamber to the top of the coverslip supports. The spirochete count reported at each observation interval was averaged from the two adjusted counts observed in both chambers. The concentration of spirochetes per mL was calculated from the average adjusted count (Figure 1).

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1. To correct for volume variability observed as depth variability between counting chambers, convert the raw number of cells counted (c_{raw}) to an adjusted number of cells counted (c_{adj}). Measure the depth of the filled chamber in micrometer graduations or focal units (Δf). Adjust by proportionality, the raw number of cells counted (c_{raw}) in a measured depth (Δf) to an adjusted number of cells (c_{adj}) in a median depth of 70 focal units (f).

$$c_{raw} + \Delta f = c_{adj} + 70f$$

$$c_{adj} = 70f(c_{raw} + \Delta f)$$

2. Compute the average (c_{ave}) of the two adjusted cell counts (c_{adj}) in a volume of 0.02 mm^3 each, obtained from an single culture, and convert the average count to number of cells per mL.

$$c_{ave}/mL = (c_{ave}/0.02\ mm^3)(50/50)(10^3\ mm^3/mL)$$

$$c_{ave}/mL = (c_{ave})(5 \times 10^4/mL)$$

Figure 1. Determination of spirochete concentration

Population doubling time determination

The average spirochete count for each observation interval was used to determine generation time. In this study, the generation time will also be referrred to as the population doubling time (PDT). The formula to calculate the PDT of bacterial cultures was available in a basic microbiology text (Volk and Wheeler 1988) and is detailed in Figure 2.

Key to symbols:

- c_o = cell concentration at start time
- c_1 = cell concentration at the first generation
- c_n = cell concentration at the nth generation
- n = the number of generations
- t_1 = time at the beginning of the observation interval
- t_2 = time of the end of the observation interval
- PDT = the population doubling time or generation time

1. If all cells survive, successive generations grow logarithmically.

   $c_1 = c_o \times 2$
   $c_2 = c_o \times 2 \times 2$
   $c_3 = c_o \times 2 \times 2 \times 2$
   $c_4 = c_o \times 2^4$
   $c_n = c_o \times 2^n$

2. To find the number of generations (n) convert all values to base log and solve for n:

   $c_n = c_o \times 2^n$
   $\log c_n = \log c_o \times n \log 2$
   $n \log 2 = \log c_n - \log c_o$
   $n = (\log c_n - \log c_o) \div \log 2$

3. The population doubling time (PDT) equals the total amount of time (Δt) between two observations (t_1 and t_2) divided by the number of logarithmic generations (n) calculated from the two observations:

   $PDT = (t_2 - t_1) \div n$
   $PDT = \Delta t \div n$

Figure 2. Determination of population doubling time (PDT)

PDT calculations were based on the observed change in spirochete concentration over time intervals between observations. The fastest PDT for one observation interval from the set of observations represented the portion of log phase growth that was the most productive. The fastest PDT intervals from all cultures were used to compare the production efficiency of the media. When more than one uncountable aggregate of spirochetes was observed in either counting chamber, the count was deemed unreliable and excluded from the results obtained in media inoculated with thawed stock. Descriptions of countable and uncountable aggregates are presented in the results. The occurrence of each type of aggregate per chamber was estimated as rare (1-2), few (3-4), some (5 to 10), or many (more than 10). When a majority nonmotile spirochetes were observed, the culture was deemed to be dying and excluded.

Media inoculated with thawed stock

The optimal volume of the inoculum consisted of a final dilution sufficient to produce a spirochete concentration near the lowest limit of detection of a Neubauer chamber, about 5 x 10^4 spirochetes/mL. Table 10 displays the inoculum dilutions applied to starting concentrations of spirochetes.

Table 10: Inoculation dilution protocol

Range of starting concentration of spirochetes (per mL)	inoculum dilution	inoculum volume (μL)
1.9 to 3.5 X 10^6	1:10	300
3.6 to 7.0 X 10^6	1:20	150
0.7 to 1.8 X 10^7	1:50	60
1.9 to 3.5 X 10^7	1:100	30
3.6 to 7.0 X 10^7	1:200	15
0.7 to 1.0 X 10^8	1:300	10

Cultures were inoculated into the same medium as well as into the various other types of medium formulations. All cultures were

incubated at 31 ± 2°C. Each culture tube was labelled with a unique accession number. The accession number was used later to correlate culture culture information such as: rodent-isolate designation, medium type and age, passage number, inoculum concentration and dilution factor, and culture dates and times.

Eight Neubauer cell counting slides containing duplicate counting chambers were available for use with a phase-contrast microscope. A batch of eight individual culture tubes were observed over the period of one week. Initially, the spirochete concentration per tube was determined every 24 hours to document the occurrence of logarithmic growth. Forty-eight hour observations were deemed sufficient for cultures with low concentrations. As the cultures became more concentrated, observations were again performed every 24 hours. Counting was terminated before one week elapsed when the cultures became too concentrated to count or when several aggregates were observed. To develop and improve quantification techniques, approximately 50 tube culture trials were conducted prior to performing tube culture tests. Eventually, the frozen stock provided a library of various isolates. Isolates were passaged exclusively in one type of medium and in more than one type of medium. The number of passages in each medium was recorded.

A batch of eight tubes was divided into duplicate tubes of standard BSK II and three sets of duplicate tubes of various modified media. For six weeks, BSK II, BSK II-H, BSK II-S, and BSK II-HS were tested in batched sets of duplicate tubes. Standard BSK II and BSK II-H medium formulations were again tested in parallel after 11 to 18 weeks storage at 2 to 8°C. For ten weeks, BSK II, BSK II-H50, BSK II-F, and BSK II-HF were tested in batched sets of duplicate tubes. The age of each medium type was recorded.

The first batch week, all eight tubes were inoculated with the same volume of an isolate suspended in BSK II medium. Subsequent batch inoculations were from either standard BSK II medium or one of the various medium modifications to nuture spirochetes grown in one or more of the various nutrient formulations.

Active passage comparisons

A fresh batch of BSK II, BSK II-H, BSK II-HS, and BSK II-S were manufactured one week prior to use for three active passage comparison experiments. Three tubes of BSK II were inoculated with B. burgdorferi isolate designated Men-W5-P3. After four days the three BSK II culture tubes were pooled and the concentration of spirochetes in the pool was measured. The BSK II pool of actively growing spirochetes was used to inoculate ten tubes each of BSK II, BSK II-H, BSK II-HS, and BSK II-S. Tube cultures were examined at 48 hour intervals over four days. Two cultures from each medium type were sampled and counted in one batch of eight counting slides. Spirochete concentration, chamber depth, observation time, and qualitative observations were recorded.

The second set of comparisons were continuous passages from the first comparison set. One tube of each medium type was used to inoculate five tubes of the same type of medium. Consequently these inoculations were nonparallel. After 48 and 72 hours quantitative and qualitative observations were performed.

The third comparison set was a repeat of the first comparison set performed using parallel inoculations. Frozen stock designate W5-P1, an aliquot from an initial BSK II culture containing an ear punch, was used to inoculate three tubes of fresh BSK II medium. After three days incubation, the spirochete concentration was measured in all three tubes. One tube was selected to be the source of all active passage inoculations into five tubes of each of the four medium types (BSK II, BSK II-H, BSK II-HS, BSK II-S). All culture tubes were inoculated with 50 μL of the active culture. All 20 tubes were observed after 48 and 96 hours of incubation. Three of five BSK II-HS and BSK II-S cultures were excluded due to culture death.

Growth from low B. burgdorferi concentration inocula

The concentration of an active B. burgdorferi culture (W5-P2) in BSK II medium was determined by averaging the adjusted counts from all eight cell counting slides. A dilution series of the active

culture in BSK II medium (1:300, 1:100, 1:10, 1:15, and 1:15) reduced the concentration of spirochetes in the inocula. Each of the last three dilutions were inoculated in five tubes of BSK II, five tubes of BSK II-H, and five tubes of BSK II-HS (Figure 3). The first parallel set of five tubes of each medium type were inoculated with 300 μL of dilute active culture broth that contained approximately 45 spirochetes. The second parallel set of five tubes of each medium type were inoculated with 200 μL of dilute active culture broth that contained approximately three spirochetes. The third set received 200 μL of dilute active culture that contained less than one spirochete. The inocula consisting of less than one spirochete were cultured to verify that the dilution series produced low B. burgdorferi concentration inocula.

Since the lowest limit of detection with a cell counting chamber was 10^5 cells/mL, a population of 10^6 spirochetes/mL was selected as the target concentration for the first observation. From an inoculum of one cell/mL, 10^6 cells/mL would be produced after twenty generations of logarithmic growth progression. At a spirochete population doubling time of 12 hours, a minimum of 240 hours (10 days) would be required to produce 10^6 spirochetes from a single spirochete. The culture tubes were first examined on day 10.

A maximum of six cultures (two from each medium type) were processed at one time. Each culture tube was vortexed gently by hand and an aliquot removed from near the bottom of the tube. However, instead of using cell counting chambers, a 50 μL aliquot from each culture was dispensed on a microscope slide and coverslipped. The slides were transported in a humidified box to avoid drying during counting. For each slide preparation, the average number of spirochetes per field (400X) was calculated from the ten fields observed. The overall average number of spirochetes within each medium set, at each inoculum level, was calculated and compared. Aggregates observed during counting were noted qualitatively. The observation protocol was repeated on day 15.

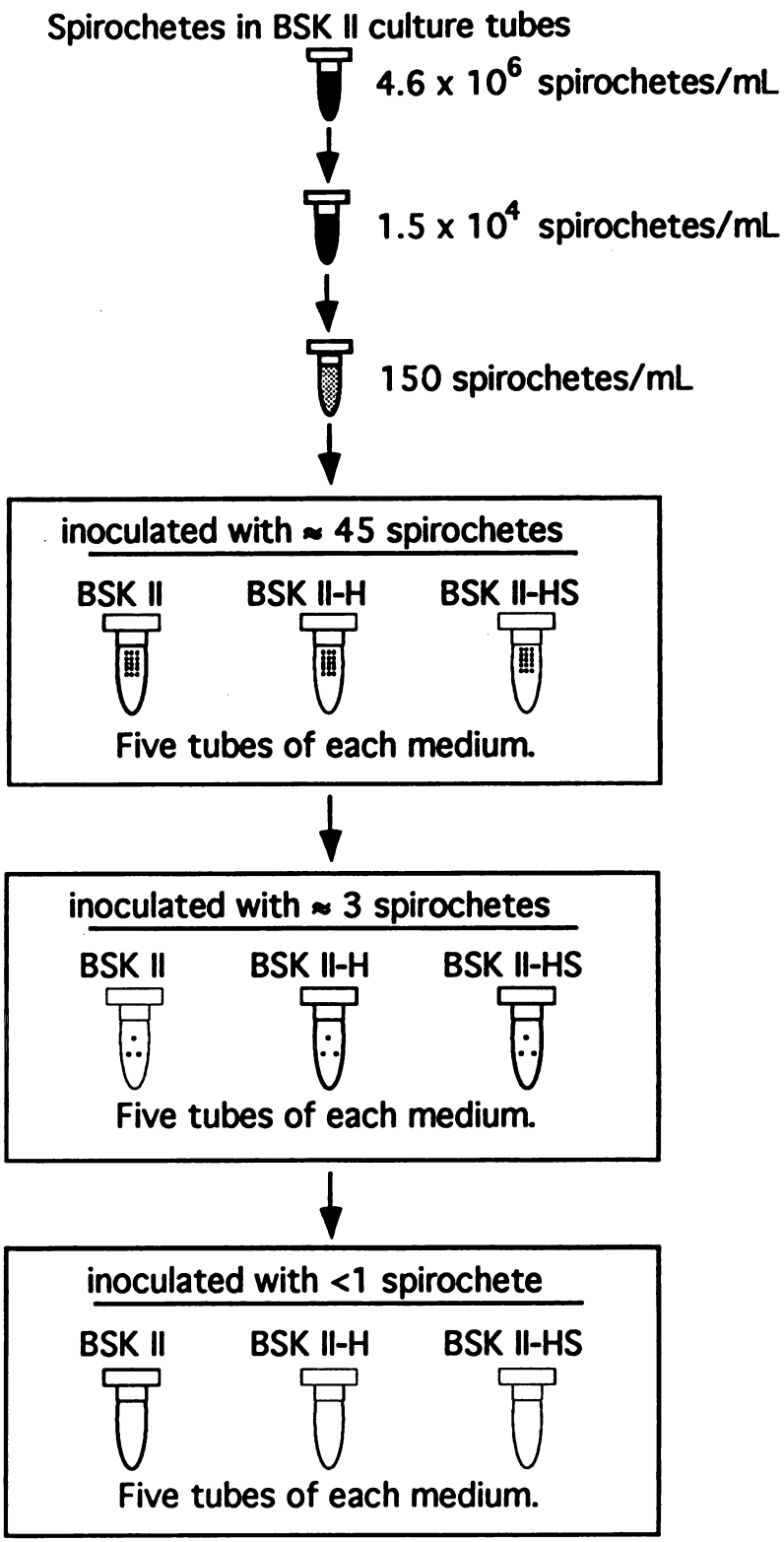


Figure 3. Serial dilution inocula diagram

Statistical analysis of results

One-way analysis of variance (ANOVA) was applied to groups of media within each growth experiment: all trials from frozen stock, parallel trials from frozen stock, parallel trials from active passages, nonparallel trials from active passage, and recovery from a low concentration of spirochetes. The software package, Systat 5 for the Macintosh, (version 5.1, 1990-91 SYSTAT Inc., Evanston, IL) performed the ANOVA calculations with Tukey HSD multiple pairwise comparisons. Probability values (p) indicated a significant difference between compared values when the probability was less than 0.050 or similarity when p was greater than 0.050. The growth parameters that were statistically compared were population doubling times (PDT's), the concentration of spirochetes between cultures inoculated in parallel, variance between culture replicates, and the variance between the two replicate experiments started by active passages from parallel inoculations.

RESULTS

Media quality control

All media produced in the laboratory passed MDPH quality control inspections. None of the basal medium aliquots nor any of the final medium products ever exhibited contamination or turbidity. All tubes retained a pinkish color. All the final medium products had pH values within the prescribed range of 7.6 ± 0.2 (Table 11).

Table 11. Medium production observed pH values

Medium type	Lot #	Manuf. date	Base pH	Final pH	QC pH
BSK II	1921	7-18-91	7.15	7.51	7.59
BSK II-H	2071	7-26-91	6.91	7.62	7.73
BSK II-H	2961	10-23-91	6.93	7.60	7.60
BSK II-HS	2961-2	10-23-91	6.93	7.60	7.66
BSK II-S	2961-1	10-23-91	7.09	7.59	7.63
BSK II-F	3441	12-10-91	7.13	7.56	7.56
BSK II-HF	3441-1	12-10-91	6.94	7.57	7.56
BSK II-H50	3441-2	12-10-91	7.09	7.57	7.56
BSK II	0942	4-10-91	7.12	7.57	NA
BSK II-H	0942-3	4-10-91	7.12	7.57	NA
BSK II-HS	0942-2	4-10-91	7.12	7.57	NA
BSK II-S	0942-1	4-10-91	7.12	7.57	NA

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-S = bovine albumin and human serum, II-HS =human albumin and human serum, II-F = bovine albumin and fetal bovine serum, II-HF = human albumin and fetal bovine serum, II-H50 = 1:1 mixture of human albumin:bovine albumin and rabbit serum.

B. burgdorferi recovery from thawed ear punches

Positive growth was obtained from ear punches within 7 to 14 days; however, a few ear punch cultures took up to three weeks incubation before exhibiting detectable growth. Often the ear punch

biopsy culture was contaminated with yeast or mold encircling the specimen. Some spirochete cultures were contaminated with a mixed flora of bacterial and fungal forms. No attempts were made to subculture and identify contaminants. Subcultures from specimen MEN-W5 were the least contaminated and used for comparative growth studies in both the active passage and the recovery experiments. Subcultures from specimen MEN-W3 were heavily contaminated throughout all passages and not used for growth comparisons despite the presence of spirochetes. All 14 of the aliquots from the glycerol-saline transport fluids taken from each of the seven storage vials were negative by culture for spirochetes but all of the stored ear punch biopsies were positive by culture.

B. burgdorferi concentration measurement

Since Borrelia burgdorferi prefers reduced oxygen conditions (Austin 1993, Barbour and Hayes 1986, Johnson, R. et al. 1984a), broth samples were collected from the bottom of the culture tubes after lightly vortexing. Culture broth samples collected from within 1 cm of the bottom of vortexed culture tubes were slightly more concentrated (range 2.5 to 44%, median 10%) than samples withdrawn from the middle or top of the same tubes. All subsequent sampling of culture broth was collected from within 1 cm of the culture tube bottom. After 72 hours incubation, the concentration of spirochetes in six undisturbed culture tubes was similar to six tubes of cultures that were gently vortexed by hand at 24 hr. intervals ($p > 0.050$).

Testing the eight Neubauer cell counting chambers for error between slides did not find appreciable differences. Table 12 lists the results obtained in both chambers of all eight counting slides prepared from the same spirochete culture. The raw counts were the actual number of spirochetes counted within five cubes (0.02 mm^3) of each chamber. The depth of each chamber preparation was measured in graduations on the fine focus knob. The adjusted counts were determined as described in the methods, Figure 1 (page 59). The average count listed was calculated from the two adjusted

counts. The overall average adjusted count for all slide preparations was 84.5 (standard deviation = 2.4, coefficient of variation = 2.9%). Table 12 also lists the deviation of each average count from the overall average count.

Table 12: Observed variation between counting chambers

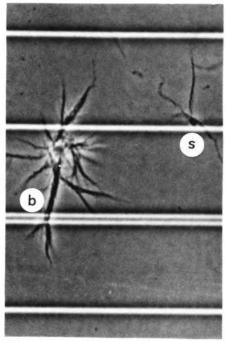
slide	chamber 1 counts			chamber 2 counts			ave.	deviation from
no.	raw	depth	adjusted	raw	depth	adjusted	count	overall ave.
1	89	70	89	77	65	83	86	+ 1.5
2	87	70	87	89	70	89	88	+ 3.5
3	81	70	81	85	65	92	86.5	+ 2.0
4	104	80	91	81	70	81	86	+ 1.5
5	85	75	79	102	85	84	81.5	- 3.0
6	78	65	84	83	70	83	83.5	- 1.0
7	100	80	88	67	60	78	83	- 1.5
8	96	80	84	73	65	79	81.5	- 3.0

Counting was terminated when samples became too concentrated ($\approx 1.3 \times 10^7$ spirochetes/mL) or when more than one uncountable aggregate was observed in one chamber. The aggregate nomenclatures used in this study (spindle, bundle, colony, and macrodeposit) reflected the size, morphology, and spirochete density of aggregated forms. The number and size of spirochete aggregates increased as a culture aged. Eventually the largest aggregate (macrodeposit) formed in the bottom of the culture tubes.

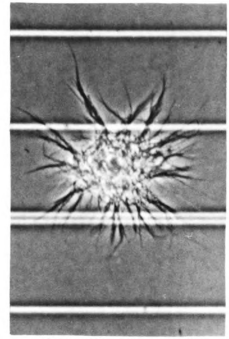
Photographs of aggregates viewed with phase contrast microscopy under 400X and 1000X magnification are in Figures 4 and 5. The single white lines (0.05 mm apart) visible in some photographs are from the etched surface of the counting chamber. A "spindle" resembled a thread of two or more entwined spirochetes. The spindle in Figure 4-1 (s) was small- to medium-sized compared to other spindle sizes observed. A "bundle" of spirochetes were connected at the middle with individual ends radiating outward resembling a bundle of sticks. The bundle in Figure 4-1 (b) was large and could also have been characterized as a small colony. Individual spirochetes in very small spindles or bundles could be

counted without too much difficulty. Sometimes the intertwined, interconnected spirochetes formed a "web" attached to the glass slide surface (Figure 4-2). A "colony" aggregate consisted of an orb-like dense mass of spirochetes surrounded by several semi-attached spirochetes giving the colony a hairy appearance (Figure 4-3 and 4-4). The colonies often had a dark, dense body centrally located within the colony. As the culture aged, the size and frequency of aggregates increased. Eventually a white deposit of cells (macrodeposit) formed in the bottom of most tubes. Figure 5-1 depicts a small macrodeposit that had formed near a much larger macrodeposit. Note the margin of this small deposit is nearly entire. Usually the margin of macrodeposits had many protruding entwined spirochetes. Figure 5-2 shows a fan-like aggregate connected to the margin of a very large macrodeposit. Rotation of the semi-attached spirochetes at the margin of large aggregates produced a current that pulled in unbound spirochetes and smaller aggregates that became incorporated into the macrodeposit. Once incorporated, the aggregates were never observed dispersing.

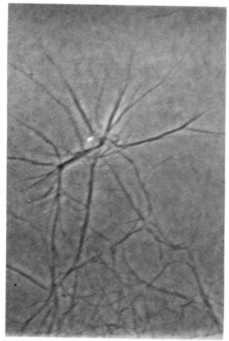
Unusual forms were occassionally observed. A "ring" formed when the ends of an individual spirochete became twisted on itself. Although closed, the spirochete continued to rotate within the ring form. Most ring forms were permanently closed but some spirochetes alternated between a ring form and its original corkscrew shape. Pairs of spirochetes were attached end to end. Other pairs resembled incomplete cell division, two ends protruding from a single, slightly larger cell cylinder. The formation of aggregates or clumps of spirochetes was not simply the product of incomplete cell division. Individual, free swimming spirochetes became entangled with one another and with spirochete aggregates. The formation of aggregates may be enhanced by the stickiness of the spirochete's surface. Spirochetes were often observed stuck to the glass coverslip and slide.



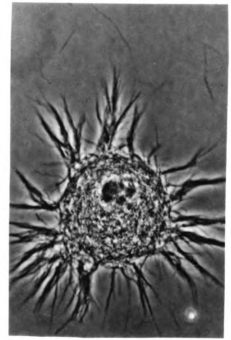
4-1. Bundle (b), spindle (s) (400X)



4-2. Large colony (400X)

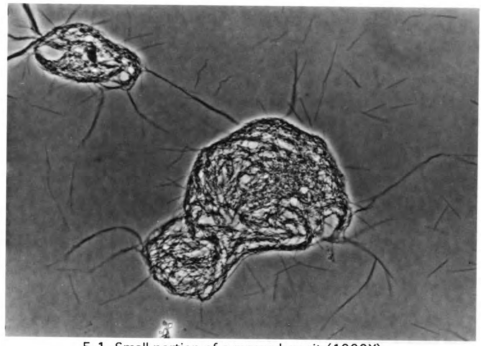


4-3. Web on glass (1000X)

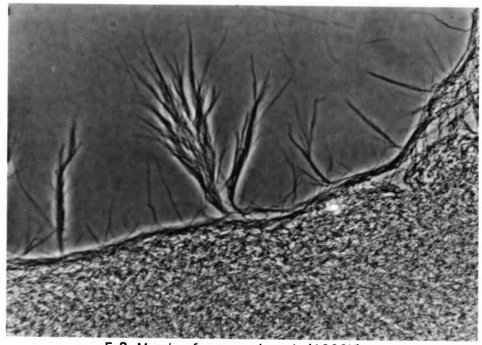


4-4. Large colony (1000X)

Figure 4. Photographs of aggregate forms



5-1. Small portion of a macrodeposit (1000X)



5-2. Margin of a macrodeposit (1000X)

Figure 5. Photographs of macrodeposits

Media inoculated with thawed stock

The fastest population doubling time (PDT) intervals usually ocurred within the first three days (72 hours) of incubation. Table 13 lists the culture medium age at inoculation, the number of trials per medium type, and the average (± 1 standard deviation) of the fastest PDT interval for all measurable intervals among cultures started from frozen stock. The results in Table 13 are a summation of parallel and nonparallel trials.

Table 13. PDT results from media inoculated with thawed stock

Medium age	Medium*	No. of trials	PDT (hours)
1-2 weeks:	BSK II-H	5	11.1 ± 2.7
	BSK II-HS	2	12.4 ± 1.9
	BSK II-H50	2	13.1 ± 0.8
	BSK II	2	13.3 ± 4.8
	BSK II-HF	2	16.6 ± 0.8
	BSK II-F	2	18.1 ± 1.8
	BSK II-S	2	25.2 ± 4.8
3-6 weeks:	BSK II-H	7	13.0 ± 1.8
	BSK II-H50	3	14.4 ± 2.9
	BSK II-HS	6	14.5 ± 2.0
	BSK II-HF	4	15.7 ± 2.4
	BSK II-S	8	20.0 ± 3.8
7-10 weeks:	BSK II-H50	5	15.3 ± 2.9
	BSK II-HF	4	17.4 ± 2.8
	BSK II-S	8	19.8 ± 3.1
	BSK II-F	5	20.0 ± 2.7
	BSK II-HS	4	21.9 ± 1.4
11-14 weeks:	BSK II	6	13.3 ± 2.2
	BSK II-H	2	14.2 ± 1.1
15-18 weeks:	BSK II-H	2	15.9 ± 0.4
	BSK II	7	18.6 ± 3.3

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-S = bovine albumin and human serum, II-HS =human albumin and human serum, II-F = bovine albumin and fetal bovine serum, II-HF = human albumin and fetal bovine serum, II-H50 = 1:1 mixture of human albumin:bovine albumin and rabbit serum.

Of the seven media tested within two weeks of manufacture, BSK II-H had the fastest average PDT (11.1 ± 2.7 hrs.), followed by BSK II-HS (12.4 ± 1.9), BSK II-H50 (13.1 ± 0.8), BSK II (13.3 ± 4.8), BSK II-HF (16.6 ± 0.8), BSK II-F (18.1 ± 1.8), and BSK II-S (25.2 ± 4.8). After noting that BSK II-H medium was capable of supporting spirochete generation times approximately equal to the standard BSK II medium, the number of culture trials in fresh BSK II-H was increased to improve statistical relevance.

Comparison of PDT results in the various media aged 1-2 weeks found only BSK II-S to be significantly different ($p < 0.050$). The remaining media supported similar PDT's ($p > 0.050$). After the media aged 15-18 weeks, the ability to support growth decreased similarly in BSK II and BSK II-H ($p > 0.050$). The PDT's produced after 18 weeks of media storage indicated a significant decrease in growth support in BSK II ($p = 0.021$) and decreased growth support in BSK II-H that approached significance ($p = 0.068$). The source of B. burgdorferi isolates varied. Some trials were excluded due to excessive aggregation or a majority of nonmotile spirochetes. The results of growth comparisons in various media inoculated from frozen stock were also evaluated as separate parallel experiments.

Parallel inoculations with thawed stock. Two of nine parallel experiments were rejected due to aggregation. The seven acceptable experiments consisted of 11 different combinations of growth comparisons between 16 media types. Eleven of the 16 media types were cultured in duplicate. In all comparisons, media formulations that contained human albumin produced higher concentrations of spirochetes than parallel media containing bovine albumin. But the fastest PDT's were evenly distributed. Of six sets of experiments that varied only the albumin component, sets 4, 5, and 6 had lower generation times in media that contained bovine albumin while sets 1, 2, and 7 had lower generation times in media formulations that contained human albumin. Table 14 summarizes the seven experiments by listing the experiment number, medium type, the number of culture tubes tested, average spirochete counts per duplicate (or individual) culture, and the average PDT calculated

from individual PDT values. The average count data with standard deviation allowed a more concise and tangible comparison of growth differences. Conversion of the average spirochete count to concentration of spirochetes per milliliter may be done by multiplying the count by a conversion factor of 5.0×10^4.

Table 14. Results from parallel inoculations with thawed stock

set#	Medium*	n	t_1 ave. count	t_2 ave. count	ave. PDT
1	BSK II-H	2	2.0 ± 0.0	125.0 ± 5.7	8.9 ± 0.1
	BSK II-HS	2	1.5 ± 0.7	28.5 ± 0.7	12.4 ± 1.9
	BSK II	2	1.5 ± 0.7	29.0 ± 15.6	13.3 ± 4.8
2	BSK II-H50	2	14.0 ± 1.4	176.5[a] ± 23.3	15.4[a] ± 1.4
	BSK II-HF	2	13.5 ± 2.1	171.5[a] ± 20.5	15.3[a] ± 1.6
	BSK II-F	2	6.5 ± 0.7	40.5[a] ± 6.4	21.5[a] ± 3.1
3	BSK II-H50	2	7.0 ± 0.0	142.5 ± 50.2	13.0 ± 1.6
	BSK II-HF	1	6.0 ± 0.0	54.0 ± 2.8	17.8 ± 0.0
4	BSK II-HS	2	8.0 ± 1.4	119.5 ± 7.8	12.6 ± 0.5
	BSK II-S	2	4.0 ± 0.0	65.5 ± 17.7	12.3 ± 1.2
5	BSK II-HS	2	103.5 ± 6.4	215.5 ± 33.2	22.9 ± 6.8
	BSK II-S	2	74.0 ± 4.2	174.5 ± 2.1	18.6 ± 1.0
6	BSK II-HF	1	63.0 ± 0.0	125.0 ± 0.0	23.8 ± 0.0
	BSK II-F	1	14.0 ± 0.0	37.0 ± 0.0	16.8 ± 0.0
7	BSK II-HF	1	45.0 ± 0.0	74.0 ± 0.0	32.7 ± 0.0
	BSK II-F	1	18.0 ± 0.0	24.0 ± 0.0	56.6 ± 0.0

[a] Some of these observations were slightly aggregated.
* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-S = bovine albumin and human serum, II-HS =human albumin and human serum, II-F = bovine albumin and fetal bovine serum, II-HF = human albumin and fetal bovine serum, II-H50 = 1:1 mixture of human albumin:bovine albumin and rabbit serum.

In set #1, the average concentrations of spirochetes at t_1 had no significant variance. At t_2, the average concentration of spirochetes per mL was significantly higher in BSK II-H (6.3×10^6) than in BSK II (1.5×10^6; $p = 0.004$) and BSK II-HS (1.4×10^6; $p = 0.004$). The average PDT's were similiar for all three medium formulations ($p > 0.050$). In set #2, the average concentration of spirochetes per mL was significantly higher in BSK II-HF at both t_1 (6.8×10^5) and t_2 (8.6×10^6) compared to BSK II-F at t_1 (3.3×10^5; p

= 0.039) and t_2 (2.0 X 10^6; p = 0.012). The average PDT's in set #2 were similar (p > 0.050).

At t_1 of set #4, comparison of the average concentration of spirochetes in BSK II-HS and BSK II-S had no significant variance. But at t_2, the difference between the average concentration of spirochetes per mL in BSK II-HS (6.0 X 10^6) and BSK II-S (3.3 X 10^6) approached statistical significance (p = 0.058). The average PDT's were similar (p > 0.050). At t_1 of set #5, the average concentration of spirochetes per mL was significantly higher in BSK II-HS (5.2 X 10^6) than the average concentration in BSK II-S (3.7 X 10^6; p = 0.032). But by t_2 of set #5, the average concentrations of spirochetes per mL in both BSK II-HS (1.08 X 10^7) and BSK II-S (8.8 X 10^6) were similar (p = 0.224). The average PDT's in set #5 were also similar (p > 0.050).

Media formulations containing the substitution of human albumin produced higher concentrations of spirochetes in sets #6 and #7 but no analysis of variance could be performed because each test consisted of one trial without replicate trials. The media formulations in set #3 vary by more than one ingredient. The BSK II-H50 medium, which contained rabbit serum and a 1:1 mixture of bovine and human albumin, improved the generation of spirochetes compared to BSK II-HF which contained fetal bovine serum and human albumin.

Cultures from active passages

The media that performed the best in culture trials from frozen stock (BSK II, BSK II-H, and BSK II-HS) were chosen to be tested further in active passage comparisons. BSK II-S was added to complement BSK II-H and BSK II-HS and provide a more complete evaluation of BSK II ingredient substitutions with human derived components. All media were manufactured on the same day, one week prior to the first set of parallel active passage comparisons. Spirochete descendants from ear punch MEN-W5 were used in all parallel and nonparallel active passage comparisons. Subcultures from this isolate had the least amount of contamination compared to

other available isolates. Isolates from MEN-W5 had also demonstrated positive growth in all medium types. The MEN-W5 subculture used in the active passage experiments had been previously passed three times in BSK II.

Parallel active passages. All media had been stored at 4°C for eight days prior to testing. The BSK II pool of active cultures used to inoculate the tubes (10 each) of BSK II, BSK II-H, BSK II-HS, and BSK II-S, had an average concentration of 4.9 x 10^6 spirochetes/mL (ave. count = 98). Considering the 1:50 inoculation dilution, a final concentration equal to 9.8 x 10^4 spirochetes/mL (ave. count = 1.98) was added to all tubes. Table 15 displays the average spirochete count, concentration, and PDT per set of ten cultures in each medium type after 48 hours incubation.

Table 15. Results from parallel active passages

Medium*	48 hr count	Concentration (per mL)	PDT
BSK II-H	188.0 ± 24	9.4 ± 1.2 x 10^6	11.1 ± 0.4
BSK II	146.8 ± 31	7.3 ± 1.6 x 10^6	11.7 ± 0.4
BSK II-HS	33.9 ± 12	1.7 ± 0.6 x 10^6	18.1 ± 1.6
BSK II-S	17.4 ± 4	8.7 ± 0.2 x 10^5	23.7 ± 3.1

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-S = bovine albumin and human serum, II-HS =human albumin and human serum.

The B. burgdorferi cultures in BSK II-H medium produced the most spirochetes and had the fastest average PDT. Pairwise comparisons of the concentration of spirochetes produced in the four medium types were significantly different ($p = 0.000$) except for the concentrations of spirochetes in BSK II-HS and BSK II-S which were similar ($p = 0.288$). BSK II and BSK II-H both supported similar PDT's ($p = 0.840$) but all other PDT comparisons between media types were significantly different ($p = 0.000$).

All ten BSK II-H tubes had a few small aggregates (3-4 per chamber). Only four aggregates were seen in all ten BSK II cultures.

Even though the BSK II-H cultures contained more aggregates than the BSK II cultures, the BSK II-H cultures produced more countable, non-aggregated spirochetes than the BSK II cultures. All BSK II-HS and BSK II-S cultures contained some aggregates (5-10/chamber).

Nonparallel active passages. All media had been stored at 4°C for 13 days. Five BSK II culture tubes were inoculated with 10 µL of an active BSK II culture that had a concentration of 8.2 X 10^6 spirochetes/mL (ave. count = 164). Five BSK II-H culture tubes were inoculated with 10 µL of an active BSK II-H culture that had a concentration of 5.85 X 10^6 spirochetes/mL (ave. count = 117). Five BSK II-HS culture tubes were inoculated with 50 µL of an active BSK II-HS culture that had a concentration of 4.9 X 10^6 spirochetes/mL (ave. count = 98). Five BSK II-S culture tubes were inoculated with 50 µL from an active BSK II-S culture that had a concentration of 3.2 X 10^6 spirochetes/mL (ave. count = 64). Three of five BSK II-S cultures were not included in the average PDT_2 calculations because the t_2 counts were less than the previous (t_1) counts. The PDT_2 were averaged from the individual counts observed between 48 and 72 hours, not from the counts for the overall life of the culture. Table 16 lists the starting spirochete count calculated at inoculation, the average spirochete counts from five tubes of each medium at 48 hours (t_1) and 72 hours (t_2) of incubation, and the average interval PDT for each set of tubes.

Table 16. Results from nonparallel active passages

Medium*	Inoc count	ave t_1 count	PDT_1	ave t_2 count	PDT_2
BSK II-H	0.39	14.4 ± 1.7	9.2 ± 0.3	76.4 ± 8.8	10.1 ± 1.4
BSK II	0.55	14.6 ± 3.1	10.3 ± 1.0	44.0 ± 21.0	18.8 ± 8.6
BSK II-HS	1.63	16.6 ± 2.6	14.5 ± 1.1	36.4 ± 5.9	21.7 ± 4.2
BSK II-S	1.06	6.8 ± 2.3	19.6 ± 7.0	11.0 ± 0.0	24.8 ± 17.0

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-S = bovine albumin and human serum, II-HS =human albumin and human serum.

Since the inoculations were not performed in parallel, the average concentrations of spirochetes were not directly compared. Although the BSK II-H cultures were inoculated with the lowest number of spirochetes, faster PDT's enabled the BSK II-H cultures to produce the highest average concentration of spirochetes after 72 hours of incubation. The average PDT's were faster in the first interval (t_1) up to 48 hours compared with the second interval (t_2) measured between 48 and 72 hours. The average PDT_1 values in BSK II, BSK II-H, and BSK II-HS were similar ($p > 0.050$). The average PDT_1 in BSK II-S was significantly slower than in BSK II ($p = 0.049$) and BSK II-H ($p = 0.029$) but similar to BSK II-HS ($p = 0.158$).

All of the average PDT_2 values were similar ($p > 0.050$). Only two BSK II-S PDT_2 values were used for statistical comparisons. BSK II-HS had the most and largest aggregates (more than 10/chamber) after both the 48 and 72 hour observations. BSK II-S also had some aggregates (5-10/chamber), followed by BSK II-H which had a few spindles and medium bundles (3-4/chamber). The BSK II cultures had rare small spindles and bundles (1-2/chamber).

Repeat parallel active passages. The first parallel active passage study, was repeated except for the age of the media which had been stored at 4°C for 16 days instead of 8 days. The BSK II culture that was the source of the parallel inoculations had a concentration of 3.3×10^6 spirochetes/mL (ave. count = 65). At an inoculation dilution of 1:50, the parallel inoculations contained approximately 6.5×10^4 spirochetes/mL (ave. count = 1.3). Three tubes of BSK II-HS and BSK II-S were discontinued at the 48 hour observation due to aggregation. The PDT_1 values at 48 hours in the repeat parallel active passage experiment were similar to the PDT_1 values at 48 hours in the first parallel active passage experiment for BSK II ($p = 0.784$), BSK II-H ($p = 0.996$), and BSK II-HS ($p = 1.000$) but the BSK II-S results were significantly different ($p = 0.000$).

Table 17 displays the average number of spirochetes counted after 48 (t_1) and 96 (t_2) hours of incubation, and the PDT values for each interval. For both observation intervals, BSK II-H had the

highest average spirochete counts and the fastest average PDT values followed by BSK II, BSK II-HS, and BSK II-S.

Table 17. Results from repeat parallel active passages

Medium*	n	ave t_1 count	PDT_1	ave t_2 count	PDT_2
BSK II-H	5	15.6 ± 2.5	13.5 ± 1.0	172.0 ± 49.4	14.1 ± 1.2
BSK II	5	9.8 ± 2.4	16.8 ± 1.9	82.6 ± 18.6	15.8 ± 2.3
BSK II-HS	2	8.0 ± 4.2	20.1 ± 6.4	38.5 ± 6.4	20.9 ± 5.0
BSK II-S	2	4.0 ± 2.8	49.5 ± 39.2	14.0 ± 5.7	25.5 ± 6.8

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-S = bovine albumin and human serum, II-HS =human albumin and human serum.

After 48 hours of incubation (t_1), the average concentration of spirochetes per mL was significantly higher in BSK II-H (7.8×10^5) than in BSK II (4.9×10^5; $p = 0.031$), BSK II-HS (4.0×10^5; $p = 0.032$), and BSK II-S (2.0×10^5; $p = 0.002$). The average concentration of spirochetes per mL at t_1 was similar in BSK II, BSK II-HS, and BSK II-S ($p > 0.050$). The PDT_1 results were similar in BSK II, BSK II-H, and BSK II-HS ($p > 0.050$). The PDT_1 results in BSK II-S were significantly different from BSK II ($p = 0.049$) and BSK II-H ($p = 0.029$) but similar to BSK II-HS ($p = 0.158$).

After 96 hours (t_2), the average concentration of spirochetes per mL was significantly higher in BSK II-H (8.6×10^6) than in BSK II (4.1×10^6; $p = 0.008$), BSK II-HS (1.9×10^6; $p = 0.004$), and BSK II-S (7.0×10^5; $p = 0.001$). The average concentration of spirochetes at t_2 was similar in BSK II, BSK II-HS, and BSK II-S ($p > 0.050$). The PDT_2 results were similar in BSK II, BSK II-H, and BSK II-HS ($p > 0.050$). The PDT_2 results in BSK II-S were significantly different from BSK II ($p = 0.018$) and BSK II-H ($p = 0.007$) but similar to BSK II-HS ($p = 0.103$). After 96 hours, BSK II-HS had many medium to large colonies (more than 10/chamber), BSK II-S had some very small bundles (5-10/chamber), BSK II-H had a few small bundles and rings (3-4/chamber), and BSK II had only a few rings (3-4/chamber) but no aggregates.

Growth from low B. burgdorferi concentration inocula

The serial dilution of the inocula compared the ability of BSK II, BSK II-H, and BSK II-HS to support growth from very low numbers of spirochetes. Prior to serial dilution, the concentration of the active culture in BSK II medium was 4.6 X 10^6 spirochetes/mL. The concentration was determined by averaging the results obtained from all eight counting slides. After serial dilution in BSK II medium, one set of five tubes of each medium type were inoculated with approximately 45 spirochetes, a second set of five tubes were inoculated with approximately three spirochetes, and a third set of five tubes were inoculated with less than one spirochete. Although each set of tubes was inoculated in parallel, statistical analysis of variance was not performed between the nonstandardized counts.

After 10 days of incubation, all media inoculated with approximately 45 spirochetes contained growth. Table 18 displays the average number of spirochetes per field (400X) in BSK II (9 ± 1.4), BSK II-H (19 ± 2.8), and BSK II-HS (2 ± 0.3). The densities of spirochetes were significantly different ($p < 0.050$). Only the BSK II-HS cultures contained some aggregates (5-10/chamber). No observable growth was detected in any of the culture tubes inoculated with three or less than one spirochete.

Table 18. Day 10 results from inoculations with 45 spirochetes

	Average number of spirochetes per 400X field from ten fields		
Culture no.	BSK II*	BSK II-H*	BSK II-HS*
1a	7.9 ± 3.3	17.8 ± 5.8	1.9 ± 1.2
2a	11.1 ± 3.7	19.5 ± 4.7	1.6 ± 1.1
3a	8.1 ± 3.4	16.4 ± 5.4	1.7 ± 1.5
4a	10.0 ± 3.4	23.9 ± 4.9	2.1 ± 1.6
5a	9.8 ± 3.0	19.6 ± 4.4	2.2 ± 1.6
overall ave.	9.4 ± 1.4	19.4 ± 2.8	1.9 ± 0.3

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-HS =human albumin and human serum.

After 15 days of incubation, the parallel BSK II and BSK II-H culture tubes inoculated with approximately 45 spirochetes had produced an innumerable amount of spirochetes in all fields but the overall average from the five BSK II-HS cultures was only 24 ± 2.1 spirochetes per 400X field (individual culture averages not shown). There were many spirochete aggregates (more than 10/chamber) observed in all microscopic fields and macroscopic deposits of spirochetes in the bottom of all culture tubes in this parallel set.

The parallel culture tubes inoculated with approximately three spirochetes exhibited greater differences in medium productivity after 15 days of incubation. Table 19 displays the average number of spirochetes per field (400X). No spirochetes were observed in the tubes designated "none."

Table 19. Day 15 results from inoculations with three spirochetes

	Average number of spirochetes per 400X field from ten fields		
Culture no.	BSK II*	BSK II-H*	BSK II-HS*
1b	none	147.2 ± 17.5	1.7 ± 1.0
2b	28.2 ± 4.8	143.7 ± 13.6	none
3b	none	none	1.8 ± 1.1
4b	none	159.4 ± 14.3	1.8 ± 0.8
5b	none	none	none
overall ave.	28.2 ± 4.8	150.1 ± 8.2	1.8 ± 0.1

* II = bovine albumin and rabbit serum, II-H = human albumin and rabbit serum, II-HS =human albumin and human serum.

One of five BSK II cultures had an average spirochete count of 28 ± 4.8 per field. No spirochetes were observed in the four remaining BSK II culture tubes. Three of five BSK II-H cultures had an average count of 150 ± 8.2 spirochetes per field. No spirochetes were observed in two BSK II-H culture tubes. Three of five BSK II-HS tubes had an average count of 2 ± 0.1 spirochetes per field. No spirochetes were observed in two BSK II-HS culture tubes. The BSK II-HS cultures contained some aggregates (5-10/chamber), even though the concentration of individual spirochetes was sparce. The

BSK II-H cultures that contained spirochetes had a few small aggregates (3-4/chamber).

No growth was detected in any of the tubes inoculated with less than one spirochete. Therefore, the serial dilutions were within the range necessary to compare growth from inocula that contained low concentrations of spirochetes. The results from the observations on day 10 are diagrammed in Figure 6. The overall average numbers of spirochetes per field are displayed below each medium tube symbol. The results from observations on day 15 are diagrammed in Figure 7. Below each medium tube symbol, are the fraction values which represent the number of spirochete positive tubes per five tubes inoculated. Below the fraction, are the overall average numbers of spirochetes per field in the positive tubes.

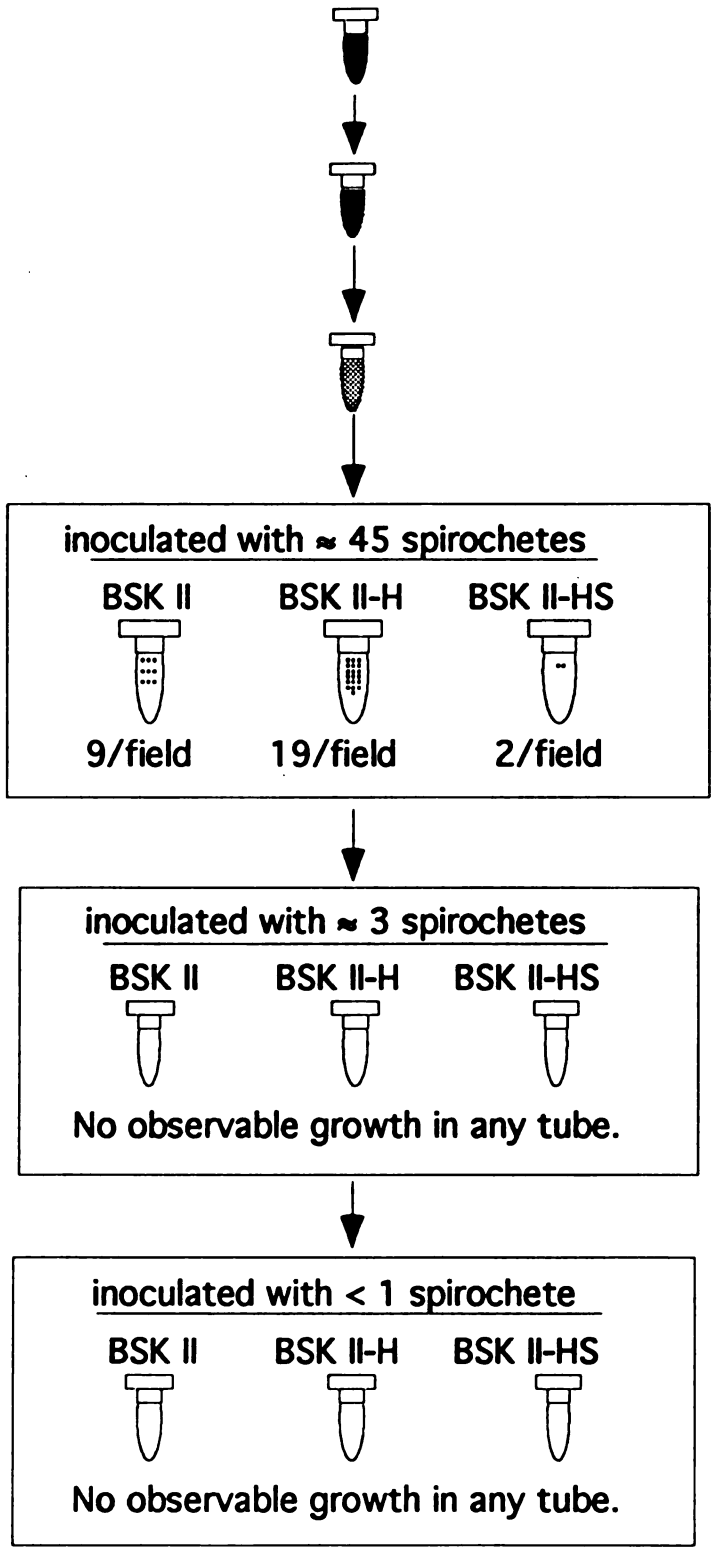


Figure 6. Growth from low concentration inocula after 10 days

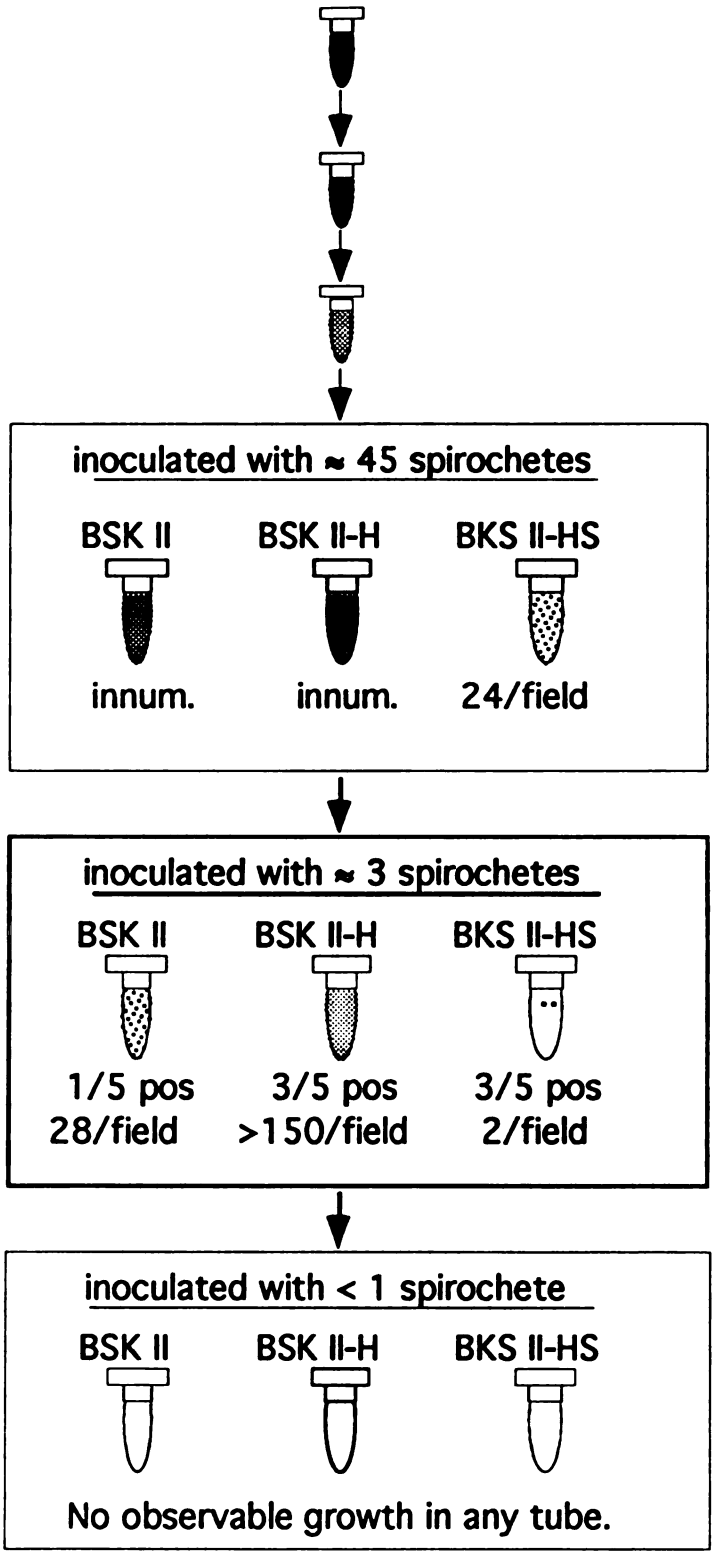


Figure 7. Growth from low concentration inocula after 15 days

DISCUSSION

This study evaluated the effect of substituting ingredients in Barbour-Stoenner-Kelly (BSK II) medium on the in vitro generation time of Borrelia burgdorferi. Substitutions for the bovine albumin component were human albumin and a combination of equal amounts of bovine and human albumin. Substitutions for the rabbit serum component were fetal bovine serum and human serum. Combinations of the albumin and serum substitutions were also tested. All test media were manufactured on the same day. In addition to testing the modified medium preparations, other variables included: the length of time the medium was stored at 4°C, the source of the spirochete isolate, the contaminants in culture, the inoculation from a thawed isolate or active passage, the concentration of spirochetes in the inoculum, the number of passages in vitro, passages from one medium type into a different medium type, the errors inherent with the method of cell counting, and the aggregation of spirochetes. These variables are discussed with greater detail below.

About 50 trial cultures, scattered across the variable continuum, were attempted before the test system was manageable and meaningful. Several of these variables were made uniform by parallel inoculation from the same spirochete culture suspension. Media inoculated in parallel attempted to equalize the effects of variations in strains of B. burgdorferi, the concentration of spirochetes in the inoculum, contamination, and the number of in vitro passages.

Apart from differences in media formulations, the variables associated with enumerating spirochetes had the most effect on generation time determinations. Much of this study hinged on the use of cell counting chambers to manually determine concentrations of spirochetes. Population doubling times (PDT's) were derived from the change in concentration over time. To reduce the dependency on

counting chambers, a parallel recovery experiment was performed.

The recovery experiment compared the ability of three medium types (BSK II, BSK II-H, and BSK II-HS) to support growth from an inoculum containing a very low concentration of spirochetes. Spirochete density was assessed by determining the average number of spirochetes per field (400X) in ten fields per culture tube. The differences in relative densities were compared to evaluate spirochete growth in the three media inoculated in parallel.

Media inoculated with thawed stock

Whether freezing helped to preserve the natural state of the spirochete or if freezing was detrimental to individual spirochetes is not known. Ewing et al. (1994) observed that freezing was detrimental to spirochete aggregates. Initial culture trials from frozen stock included comparisons of media types, sources of isolates, and inoculum volumes. All trials from thawed stock were cultured in duplicate tubes. Some of the growth data from frozen stock in fresh media were excluded due to aggregation. The growth data from the spirochete cultures inoculated with thawed stock was collated two ways. The first method compiled all culture PDT's according to the age of the media, regardless of possible isolate strain variations. The second method compared the average PDT's in cultures inoculated in parallel. The result of all PDT comparisons found that BSK II-H medium which contained human albumin supported the growth of B. burgdorferi as well or better than BSK II medium which contained bovine albumin. Average PDT's had greater variability in BSK II cultures than BSK II-H cultures which indicated that BSK II-H supported growth more consistently than BSK II. When combined with substitutions of serum, media that contained human albumin generally decreased the generation time compared to similar substitutions of serum combined with bovine albumin.

Among media aged 1-2 weeks, the fastest average PDT was obtained in BSK II-H (11.1 ± 2.7) followed by BSK II-HS (12.4 ± 1.9), BSK II-H50 (13.1 ± 0.8), BSK II (13.3 ± 4.8), BSK II-HF (16.6 ± 0.8), BSK II-F (18.1 ± 1.8), and BSK II-S (25.2 ± 4.8). All of the PDT values

were similar ($p > 0.050$) except for the PDT in BSK II-S which was significantly different from all other PDT values ($p < 0.050$). The number of cultures in fresh BSK II-H medium was increased when preliminary data indicated that B. burgdorferi growth support in BSK II-H was equivalent to BSK II medium. Media inoculated in parallel with thawed stock provided more direct comparisons than a summary comparison all cultures started from thawed stock. In parallel comparisons varying only one component, the media that contained human albumin produced more spirochetes than media that contained bovine albumin. The PDT values within parallel sets of duplicate cultures were similar ($p > 0.050$).

The overall numbers of cultures in fresh media aged 1 to 2 weeks were low due to a combination of factors. Increased production of very low volumes of fresh media (50 mL for example) was not practical. To avoid wasting media, reagents, and expensive filters, media production was limited to four occasions. The data for BSK II and BSK II-H aged 7 to 10 weeks was absent because during this time, the active passage comparisons were in progress. BSK II and BSK II-H media aged 11 to 14 and 15 to 18 weeks in 4°C storage were evaluated by inoculation with thawed stock. The number of cultures in aged BSK II was increased to evaluate the effect of medium age on the ability to support B. burgdorferi growth.

The effect of medium age on B. burgdorferi growth

The length of time that the media were stored at 4°C had an effect on the PDT's of B. burgdorferi in cultures from thawed stock. After 15-18 weeks of storage at 4°C, the average PDT in BSK II medium was significant decreased ($p = 0.021$). The average PDT in BSK II-H stored for four months also lost some ability to support B, burgdorferi growth ($p = 0.068$). Aged medium was not tested for its ability to recover spirochetes from inocula containing low concentrations of spirochetes. Chemical analysis of nutrient availability and degradation over time was not performed. Although fresh medium is desirable, it was not necessary for optimal growth support. After four months of storage, consideration should be given

to restocking with fresh medium. The shelf life of the medium has not been standardized. Dr. Stoenner et al. (1982) reported that the multi-nutritional supplement CMRL 1066 gradually lost its ability to enhance spirochete growth it neared the six month expiration date.

Active passage comparisons

Active passage comparisons were conducted with BSK II, BSK II-H, BSK II-HS, and BSK II-S. All media were produced on the same day. Spirochete isolate MEN-W5, grown in BSK II, was used for all culture comparisons. The stimulation of spirochete growth by passage from BSK II into a different medium may account for the improved growth observed in BSK II-H. But the nonparallel active passage experiment used spirochetes grown in the same medium type as that being inoculated and BSK II-H still supported growth better than BSK II.

Parallel active passages. The first parallel experiment was conducted in 10 tubes of each of the four medium types. The PDT's in BSK II and BSK II-H were similar ($p = 0.840$) but all other pairwise PDT's comparisons between media types were significantly different ($p < 0.050$). Slower PDT's were observed in BSK II-HS and BSK II-S. These results were similar to those obtained in media inoculated with thawed stock. The substitution of human serum slowed spirochete growth but growth was improved when combined with the substitution of human albumin for bovine albumin.

The average concentration of spirochetes per mL was significantly greater in BSK II-H than in BSK II ($p = 0.000$). The average concentration of spirochetes per mL was similar in BSK II-HS and BSK II-S ($p = 0.288$). Despite the possible adverse effect of aggregation sequestering countable spirochetes in BSK II-H cultures, higher counts were obtained in BSK II-H than in BSK II. Large aggregates were present in the BSK II-HS and BSK II-S cultures. The cultures were discontinued after 72 hours when macroscopic deposits were visible in the bottom of the culture tubes.

Nonparallel active passages. Nonparallel active passages were conducted using an active spirochete culture from each medium type to inoculate five tubes of the same type of medium. Consequently the initial concentration of spirochetes was not the same between medium types. Applying the inoculation dilution factor to the last observed count, the five tubes of BSK II-H started with slightly less spirochetes than the five tubes of BSK II. The concentrations of spirochetes could not be directly compared between nonparallel cultures. Media performance comparisons were based on PDT values.

After 48 hours, the average concentration of spirochetes per mL was equivalent in the BSK II-H and BSK II cultures. The average PDT_1 up to 48 hours was similar in BSK II-H and BSK II ($p = 0.967$). The PDT_1 in BSK II-HS was similar to BSK II, BSK II-H, and BSK II-S ($p > 0.050$). The PDT_1 in BSK II-S was significantly different from BSK II and BSK II-H ($p < 0.050$). Although the nonparallel 48 hour counts were equivalent in BSK II and BSK II-H, after 72 hours, the multiplication of spirochetes in BSK II-H surpassed BSK II.

After 72 hours of incubation, all of the average PDT_2 values were similar ($p > 0.050$) Three of five BSK II-S cultures were excluded from the 72 hour results because the 72 hour counts were lower than the 48 hour counts. Consistent with earlier comparisons, the medium combinations containing human serum supported growth better in the presence of human albumin instead of bovine albumin. After the nonparallel experiment, a repeat parallel active passage experiment was performed using one active BSK II culture to inoculate five tubes of each of the four types of media.

Repeat parallel active passages. Spirochete growth in BSK II-H surpassed growth in BSK II at both the 48 and 96 hour observations. After 48 hours, the average concentration of spirochetes per mL was significantly higher in BSK II-H than in BSK II ($p = 0.031$), BSK II-HS ($p = 0.032$), and BSK II-S ($p = 0.002$). The average concentration of spirochetes per mL in BSK II, BSK II-HS, and BSK II-S were similar ($p > 0.050$). The average PDT_1 values in BSK II-H, BSK II, and BSK II-HS were similar ($p > 0.050$). The average PDT_1 in BSK II-S was

significantly slower than in BSK II-H ($p = 0.029$) and BSK II ($p = 0.049$) but similar to BSK II-HS ($p = 0.158$).

After 96 hours, the average concentration of spirochetes per mL was significantly higher in BSK II-H than in BSK II ($p = 0.008$), BSK II-HS ($p = 0.004$), and BSK II-S ($p = 0.001$). The average concentration of spirochetes per mL in BSK II, BSK II-HS, and BSK II-S was similar ($p > 0.050$). The average PDT_2 values in BSK II, BSK II-H, and BSK II-HS were similar ($p > 0.050$). But the average PDT_2 in BSK II-S was significantly slower than in BSK II ($p = 0.018$) and BSK II-H ($p = 0.007$) but similar to BSK II-HS ($p = 0.103$).

The BSK II-H cultures contained small bundle aggregates, some of which were not countable, but no aggregates were observed in the BSK II cultures. Although many large aggregates were observed in the BSK II-HS cultures and only very small bundles were observed in the BSK II-S cultures, the average spirochete count in BSK II-HS was greater than in BSK II-S. Three cultures of BSK II-HS and three cultures of BSK II-S were excluded from the 96 hour data.

Growth from low *B. burgdorferi* concentration inocula

Medium performance was also assessed by inoculating BSK II, BSK II-H, and BSK II-HS with very low numbers of spirochetes obtained from a dilution series of an active culture in BSK II. Parallel sets of five tubes of each medium were inoculated with either 45 spirochetes, three spirochetes, or less than one spirochete. Although the exact numbers of spirochetes may not have been achieved by dilution, the method provided inocula that contained low numbers of spirochetes. All tubes in each set were inoculated from the same dilution suspension. After 10 and 15 days of incubation, the average number of spirochetes per field (400X) was determined from 10 fields for all culture tubes that containined spirochetes. The average number of spirochetes per field were significantly different between the three media ($p < 0.050$).

An incubation period of 10 days allowed detectable numbers of spirochetes to develop in the parallel tubes inoculated with 45 spirochetes. The BSK II-H cultures averaged 19 $\pm$ 2.8 spirochetes

per field, the BSK II cultures averaged 9 ± 1.4 per field, and the BSK II-HS cultures averaged 2 ± 0.3 per field. Spirochete aggregates were observed in the BSK II-HS cultures but not in the BSK II or BSK II-H cultures. Growth was not detected in tubes inoculated with either three spirochetes or less than one spirochete.

After 15 days of incubation, the cultures inoculated with 45 spirochetes and previously ennumerated at 10 days, had produced uncountable suspensions of spirochetes in BSK II and BSK II-H. BSK II-HS averaged only 24 ± 2.1 spirochetes per field. BSK II-HS also contained many large aggregates. Among the tubes inoculated with three spirochetes, spirochete growth was observed in three tubes of BSK II-H, three tubes of BSK II-HS, and one tube of BSK II. The BSK II-H cultures averaged 150 ± 8.2 spirochetes per field, the BSK II culture averaged 28 ± 4.8 per field, and the BSK II-HS cultures averaged 2 ± 0.1 per field. The number of positive tubes should not be used to directly compare medium performance. Some of the negative tubes may not have been inoculated with a spirochete. However, the occurrence of no growth among these tubes reaffirms that the dilution series was effective in providing low concentration inocula. Many aggregates were observed in the BSK II-HS cultures even though the concentration of individual spirochetes was sparse.

Substitutions of albumin

The medium formulation containing human albumin, BSK II-H, improved the population doubling time and the recovery of B. burgdorferi from inocula that contained low concentrations of spirochetes. The substitution of human albumin also improved the poor performance of media that contained bovine albumin and substitutions of sera. Average PDT calculations for BSK II-H had smaller standard deviations which indicated more consistent performance. The bovine albumin used in this study was from the same lot. The human albumin used in this study was a mixture of small portions from several lots. The lot mixture may have contributed a broader spectrum of nutrients to the media that contained the human albumin. Although the commercially prepared

bovine albumin had not neared the expiration date, the length of time in storage and possible decrease in potency was not known.

Callister et al. (1990) reported that the ability of BSK II medium to recover B. burgdorferi from decreasing concentrations of spirochetes in the inocula varied between lots of bovine albumin obtained from the same manufacturer and from different manufacturers. Bovine albumin variability also caused observable morphologic differences and influenced the quality of an indirect fluorescent antibody test (Callister et al. 1990). Increasing the amount of bovine albumin added by 40% in a modified medium (MKP) did not significantly improve spirochete growth (Preac-Mursic et al. 1986 and 1991). A serum-free medium supported spirochete growth better when a crude form of albumin was added instead of high grade albumin (Barbour 1986).

Substitutions of sera

The rabbit serum ingredient in BSK II was heat inactivated, trace hemolyzed, and filter sterilized. The substitutions of sera were filter sterilized prior to addition. The BSK II-S and BSK II-HS media used in culture trials inoculated with thawed stock, contained human serum that was not heat inactivated. Heat inactivation to remove complement activity may be irrelevant. B. burgdorferi has been shown to resist complement activity in the absence of a specific antibody (Kochi et al. 1991). None of the serum components were tested for the presence of antibodies directed against B. burgdorferi. The BSK II-S and BSK II-HS media used for comparisons among active passages and for growth from low spirochete concentration inocula, contained human serum that was heat inactivated. The fetal bovine serum was heat inactivated. The amount of trace hemolysis in each serum component was distinctly different. The rabbit serum contained trace hemolysis and was red-amber in color. The fetal bovine serum was only slightly hemolyzed and had a faint pink color. The human serum, clearly yellow in color, had no hemolysis. The absence of red cell components may have reduced nutrient availability. B. burgdorferi produces a hemolysin

(Williams and Austin 1992). The purpose of the hemolysin may be defensive, invasive, or to degrade extracellular nutrients that promote spirochete growth

Williams and Austin (1992) observed the hemolytic activity of three isolates of B. burgdorferi on variants of solid BSK II medium containing rabbit serum and blood from various sources. Based on the size of hemolysis zones, the horse erythrocytes were most susceptible to the borrelial hemolysin, followed by bovine, sheep, and least of all, rabbit erythrocytes. Clear zones of hemolysis were observed with the unaided eye after eight days of incubation on plated medium containing horse erythrocytes. After two weeks the entire plate was hemolyzed. However plated medium that contained citrated human blood failed to support the growth of one isolate (strain Sh-2-82) and slowed the growth of type strain B-31 so that three to four weeks of incubation produced only pinpoint zones of beta-hemolysis (Williams and Austin 1992). B. burgdorferi growth was decreased significantly when rabbit serum was used to replace fetal bovine serum in a tissue culture medium (LB-15B) during cocultivation with tick cells (Kurtti et al. 1988).

In this study, the BSK II-S cultures that contained human serum had decreased spirochete growth consistent with the reduced growth observed by Williams and Austin (1992) on a solid medium that contained citrated human blood. The lack of available nutrients from hemolyzed components in the human serum used in BSK II-HS and BSK II-S may have reduced B. burgdorferi growth. However, a component present in the human serum may have inhibited spirochete growth. While serum-free medium was not tested, the addition of serum to BSK II medium has been shown to be more beneficial to spirochete growth (Åsbrink and Hovmark 1985, Austin 1993, Pollack et al. 1993).

Sources of B. burgdorferi spirochetes

B. burgdorferi spirochetes were grown from seven of seven thawed ear punch biopsies that had been thawed once and stored at -70°C in 30% glycerol-saline solution for 8 to 18 months. Skin

punch biopsies used to initiate spirochete cultures were cultured in each unique medium type. It was possible that growth differences existed because the various B. burgdorferi isolates had different phenotypes or genotypes that enhanced or reduced growth. Different strains of B. burgdorferi do exist within the endemic area in the Upper Penninsula of Michigan from which these isolates were collected (Dr. Barbara Robinson-Dunn, manuscript in preparation). Mixed strain infections have also been detected (Demaerschalck et al. 1995). Growth differences by isolate were not compared. The possibility that different strains were used in this experiment was not determined. Strain variation would have influenced only the nonparallel cultures inoculated with thawed stock. The same isolate was used for all culture comparisons in the active passages and the growth from low B. burgdorferi concentration inocula.

Contaminants

Stringent quality control during medium production and regular testing of randomly selected culture tube blanks checked medium purity. At no time did quality control testing or a culture blank develop evidence of contamination. Despite these measures and sterile specimen transfer techniques, all cultures contained some degree of contamination. Most likely the contaminants originated from the hairy surface of the ear punch biopsies or from within the biopsy tissue. Other researchers have dipped ear biopsies in disinfectants to sterilize the hairy skin surface (Campbell et al. 1994, Oliver et al. 1995). The effects of surface sterilization on the spirochetes in the biopsy is not known and the procedure has not been standardized. In this study, surface sterilization of the thawed ear biopsies was not performed. A contaminated culture never became less contaminated after passage into fresh medium. Heavily contaminated cultures started from positive ear punch biopsies did produce spirochetes but were not used for media comparisons. Future work to optimize medium performance should include improving antibacterial and antifungal components to eliminate or reduce persistent contaminants.

The presence of contaminants in B. burgdorferi cultures has been reported (Barthold et al. 1991, Mitchell et al. 1994, Pachner et al. 1993, Pollack et al. 1993, Sinsky and Piesman 1989). Antibiotic resistant contaminants are difficult to eliminate from a liquid culture medium. Physical separation of the spirochetes from the contaminants has been proposed by culturing on solid media with a reduced oxygen atmosphere (Preac-Mursic et al. 1991). Separation of spirochetes from bacterial contaminants might also be accomplished by filtration provided the spirochetes will migrate throught the filter. Separation was reported by forcing an aliquot of a mixed bacteria culture in BSK II that contained B. burgdorferi through either a 0.45 or 0.2 μm filter and dispensing the filtered suspension into fresh BSK II (Jobe et al. 1993). The most effective means of reducing contamination may be improved sterilization of the tick or tissue specimen prior to in vitro cultivation.

Number of culture passages

B. burgdorferi cultures in BSK II medium undergo detrimental changes after approximately 15 passages. (Barbour 1988b, Moody et al. 1990, Schwan et al. 1988a, Wilske et al. 1988) In this study, only 6 of the 88 cultures inoculated from frozen stock had been passaged five times with -70°C freezer storage between passages. Only 2 of the 88 cultures were passaged four times. All the remaining culture tests started from frozen stock were passaged a maximum of three times. The isolates used in the active passages and growth from low spirochete concentration inocula were passaged a maximum of three times. There was no evidence that five passages affected the growth results.

Variables encountered during inoculation procedures

Since B. burgdorferi prefers microaerophilic conditions, attempting to distribute the spirochetes throughout the medium by vortexing may have had a detrimental effect by increasing the air content of the medium surrounding the spirochetes. Daily, gentle

vortexing by hand did not have a significant effect on growth in six culture tubes vortexed every 24 hours compared with six undisturbed tubes ($p > 0.050$). The distribution of spirochetes throughout the total volume of medium at the time of sampling was not uniform. Broth samples collected within 1 cm of the bottom of vortexed culture tubes were slightly more concentrated (range 2.5 to 44%, median 10%) than samples from the middle or top of the tubes. Therefore, all cultures were gently vortexed by hand prior to sampling and then sampled within 1 cm of the bottom of the tube.

Generation times during the first observation interval in media inoculated with thawed stock may have been prolonged by a lag time needed for the spirochetes to revive. This lag time would artificially lengthen the initial generation time calculated by PDT. Ewing et al. (1994) observed that the aggregate clumps present in low passage isolates were unable to survive freezing. Spirochete mortality or reduction in virulence due to freezing was not determined.

There were some observations that indicated B. burgdorferi growth was stimulated by transferring a culture grown in one variant of BSK II medium to another medium variant. To assess this possibility, culture comparisons must have been inoculated in parallel and contain control cultures in the medium with the same formulation as the inoculum. Cross inoculations should also be performed among the same set of media to fully compare the effect on spirochete growth that might be exerted by stimulation from a change in medium conditions. Stimulating bacterial growth by changing the nutritional environment is a well known fact. However, not enough comparisons that fit the above criteria were available to evaluate correlations between nutrient change and stimulation.

The formation of mixed medium conditions in test cultures was produced by the transfer of medium suspension included with the inoculum. Washing the spirochetes in PBS prior to inoculation was not done because formation of a pellet would have enhanced aggregation that does not readily disperse. In some culture trials with thawed stock, the inoculation dilution was only 1:10 but for all active passage trials, the inoculation dilution was never less than

1:50. The nonparallel active passages were transferred into the same medium type, which reduced mixed nutrient conditions. The inoculation dilutions in the recovery experiment consisted of three levels, 1:10, 1:15, and 1:15. The possible relationship between increasing mixed nutrient conditions and improved spirochete growth was not evaluated. BSK II-H50 contained a 1:1 ratio of bovine and human albumin. Among media aged 1-2 weeks, the spirochete growth in BSK II-H50 was similar to the growth in BSK II ($p = 0.717$) and BSK II-H ($p = 0.652$). The volume of the inoculum was designed to apply a dilution factor to the starting concentration of spirochetes so that the increase in spirochete concentration during the observation intervals would be measurable with the cell counting chambers.

Transfer of spirochete aggregates within the inoculum may have artificially increased the initial spirochete counts and initial PDT's if a significant number of spirochetes were able to disperse from the aggregate. But the dispersal of an aggregated spirochete to a free swimming, countable spirochete was never observed. Since free swimming spirochetes became aggregated and tended to stay aggregated, the presence of aggregates in the inoculum probably sequestered spirochetes and artificially reduced spirochete counts and prolonged PDT's.

B. burgdorferi concentration measurement

The majority of researchers enumerating B. burgdorferi utilize either Neubauer or Petroff-Hauser cell counting chambers. Only certain cell counting chambers were capable of functioning under phase contrast illumination. Those that performed the best were "Bright Line" (AO American Optical Corp., Southbridge, MA) with a flat undersurface and the "Improved Neubauer" (Max Levy Autograph, Inc., Philadelphia, PA) with a concave undersurface. The same counting chamber was used to determine spirochete concentration for an individual culture over time. This further reduced variability inherent in using different counting chambers to measure the same culture. There are two cell counting chambers per slide. Both

chambers were prepared from the same culture tube, so that counting was performed in duplicate, averaged, and recorded for that observation. Manual spirochete counts were performed in a total of five cubes (0.02 mm^3) in each chamber. The counts in both chambers of the counting slide were averaged and recorded. A comparison of spirochete counts between counting slides filled from a single culture tube resulted in no appreciable variation (coefficient of variation = 2.9%). The cell counting slide method of determining spirochete concentration had a limited measurable range for enumerating spirochetes.

The lowest measurable spirochete concentration was 2.5×10^4 spirochetes/mL (one spirochete per 10 cubes). To reduce low limit error, observed counts less than 1.0×10^5 spirochetes/mL (two per five cubes) were not used to determine spirochete concentration in media trials from thawed stock. The upper limit of measurable concentration was dependent on the ability to distinguish individual spirochetes in highly concentrated cultures. Concentrations greater than 7.5×10^6 spirochetes/mL (30 per cube) were difficult to accurately count. Concentrations greater than 1.25×10^7 spirochetes/mL (50 per cube) were excluded due to the loss of counting accuracy. Highly concentrated samples could have been diluted prior to transfer to a counting chamber. However, the increased presence of aggregates probably would preclude the need for predilution. Performing direct counts on eight preparations took one to two hours depending on the spirochete concentration.

Dispensing culture aliquots with large bore disposable pipets by capillary action into counting chambers tended to overfill the chambers. The overfill caused the cover glass to float which varied the depth of the culture sample between counting chambers. When the depth of the prepared chambers varied, the volume also varied which affected the spirochete count in each chamber. Therefore, an effort was made to control this variability by adjusting the count with a proportional correction performed to make the sample volumes more uniform. The adjustment was based on the height of the coverslip supports measured in fine focus graduations for all of the counting chambers. The proportional adjustment compensated

for variations in chamber preparation volumes. Future culture sampling should use microcapillary pipets with slower capillary fluid flow into the counting chambers which helps to prevent overfill. The microcapillary pipets must be capable of sampling from the bottom of the culture tubes.

While many researchers have used cell counting chambers to quantify B. burgdorferi spirochetes in vitro, none have elucidated on their method of counting spirochetes nor detailed enumeration difficulties other than aggregation (Callister et al. 1990, Dever et al. 1992, Hofmeister et al. 1992, Kurtti et al. 1987 and 1993, Preac-Mursic et al. 1991). Technical improvements are needed because of the difficulties encountered while attempting to count spirochetes swimming in and out of focus while scanning through a 0.1 mm thick culture suspension. Staining or immobilizing the fast moving spirochetes would prohibit evaluating viability based on motility. Two separate slide preparations, one to measure spirochete motility and one to fix and stain the spirochetes for enumeration, would be more labor intensive. Staining would enhance visibility, increase counting accuracy, and may improve counting chamber applications.

Initially, growth was measured every 24 hours starting from the time of inoculation and continuing for about one week. Since the most rapid growth occurred within the first few days following inoculation, 12 hour reading intervals for three or four days were most efficient. Shortening the total observation period to four days may reduce interference by formation of spirochete aggregates.

When the spirochete growth comparisons were confined to parallel cultures, the relative number of spirochetes per magnification field provided sufficient evaluation. Callister et al. (1990) averaged spirochete counts in 25 fields using darkfield microscopy and a 10 μL aliquot of culture. Growth comparisons were then made between culture tubes. A similar method of comparing spirochete densities was used in this study to compare growth from low B. burgdorferi concentration inocula. A 50 μL aliquot of culture was placed on a microscope slide and coverslipped. Under 400X magnification, the average number of spirochetes per field was determined from observation of 10 fields.

The average number of spirochetes per field were then compared between the cultures inoculated in parallel to evaluate the ability of the media to support growth. A micro-gridded coverslip, such as CELLocate™ (Eppendorf Scientific, Inc., Madison, WI) would improve this technique of spirochete enumeration.

Comparisons of population doubling times

Peak culture concentration of B. burgdorferi occurred within four to six days after inoculation and was determined to be between 10^6 and 10^8 spirochetes/mL (Preac-Mursic et al. 1991). Strain B31 was observed to enter early stationary phase at a concentration of 0.5-1 X 10^8 spirochetes/mL (Barbour et al. 1983). The observed concentration range in this study was approximately 10^5-10^7 spirochetes/mL which probably included at least the late log phase of growth prior to the stationary phase. However, the most rapid growth phase may have occurred at concentrations below the measurable range when nutrients were most abundant and there was no accumulation of toxic by-products. Conversely, the spirochetes growth rate may be maximized only after conditioning of the medium. Development of suitable biochemical conditions would be analogous to conditioning aquarium waters before the fish thrive.

Selecting a specific PDT interval to report as representative for a test culture was not always straightforward. To get an accurate measure of the generation time, the reported PDT for an observation interval had to pass four criteria. First, to represent the log phase of growth, the fastest PDT interval was selected for comparison with other cultures. Second, the spirochete count had to be within the limits of counting to maintain accuracy. The low limit was 1.0 x 10^5 spirochetes/mL. The high limit was 1.25 X 10^7 spirochetes/mL. Third, PDT's derived from cultures containing many nonmotile spirochetes were rejected. Finally, culture samples that contained more than two uncountable aggregates per chamber were not considered to be accurate for that observation interval.

To compare the abilities of the media to support growth, the PDT reported for a particular culture was the fastest value

determined for any interval in the set of observation intervals for that culture. The PDT's from observation intervals of a particular culture delineated slope portions of the growth rate curve. The fastest PDT interval represented the steepest slope (log phase) of the growth curve. When comparing growth rates, it would not be appropriate to average in portions of the lag phase and stationary plateau. Similarly, an overall PDT averaged from more than one observation interval would lengthen the fastest PDT interval by averaging in slower PDT intervals. Plotting the PDT's per culture over time, produced very few sigmoidal growth curves (data not shown). Most growth curves were nearly linear with a positive slope of varying amplitude. Some growth curves had a plateau in the middle which indicated biphasic growth or possibly a decrase in spirochete count due to an aggregation effect. A few curves were flat or had a negative slope indicative of a dying culture. Dying cultures or biphasic growth may have been caused by the presence of a predatory phage virus (Barbour and Hayes 1986, Hayes et al. 1983). It was not possible to consistently correlate shortest PDT and either spirochete concentration or incubation time. Enumeration of individual spirochetes to derive PDT's did not allow monitoring generation time after aggregates formed. Consequently it was not known whether the maximum growth rate occurred before or after the formation of aggregates.

At very low concentrations of spirochetes, small differences in either the number of spirochetes counted or the observation time, produced noticeable differences in the PDT's. Table 20 displays an example of PDT values that were affected by a small difference in spirochete counts from parallel culture tubes. Despite the same count at the 74 hour observation, a difference of one spirochete in the initial (43 hour) counts from parallel cultures #153 and #154 was magnified as a PDT difference of 5.7 hours. Therefore, large differences in PDT comparisons at low spirochete concentrations might not reflect large differences in medium performance.

Table 20: PDT variance among low concentration observations

tube id	43 hr count	74 hr count	time interval	PDT
BSK II-HF #153	3	10	31 hrs	17.8
BSK II-HF #154	4	10	31 hrs	23.5

A high spirochete count should indicate optimal medium performance but this would not be reflected by the PDT if the subsequent count did not continue to be substantially elevated. Table 21 displays an example of this phenomenon that occurred in the spirochete counts from parallel culture tubes. The count comparisons were comparable except for the t_3 count from culture #154 which was much higher than culture #153. Culture #154 had a higher final concentration of spirochetes and the fastest PDT interval (PDT_2 at 10.0 hrs.). But culture #153 had a better PDT_3 (25.4 hrs.) and a better overall PDT at 27.8 hours compared to 29.0 hours in culture #154. When the protocol to report the fastest PDT interval as representative of log phase growth was applied to the results in Table 21, the PDT for culture #153 was 15.1 hours versus 10.0 hours for #154. Even though the difference in final counts was only 16 spirochetes, the difference in reported PDT's was 5.1 hours.

Table 21: A single high count effect on PDT determination

	t_1	t_2		t_3		t_4		overall
tube designation	count	count	PDT_1	count	PDT_2	count	PDT_3	PDT
BSK II-HF #153	3	10	17.8	33	15.1	108	25.4	27.8
BSK II-HF #154	4	10	23.5	61	10.0	124	42.5	29.0

Table 22 displays the results from three sets of cultures inoculated in parallel with thawed stock. The PDT results did not correlate with spirochete productivity. The lack of correlation may have been caused by the effect of very low counts on PDT determination (previously discussed with Table 20). Media set #4 in

Table 22 shows that although the BSK II-HS cultures averaged twice as many spirochetes than the BSK II-S cultures at the initial observation and nearly twice as many at the final observation, the results of calculated PDT's favored the less concentrated BSK II-S cultures. Media sets #5 and #6 show other noncorrespondence of increased spirochete concentrations and reported PDT values among cultures inoculated in parallel. These results indicate that the spirochete growth rates in the more dense tubes may have peaked prior to the observation interval.

Table 22: Noncorrespondence of spirochete production and PDT

set #	Medium	n	t_1 ave. count	t_2 ave. count	ave. PDT
4	BSK II-HS	2	8.0 ± 1.4	119.5 ± 7.8	12.6 ± 0.5
	BSK II-S	2	4.0 ± 0.0	65.5 ± 17.7	12.3 ± 1.2
5	BSK II-HS	2	103.5 ± 6.4	215.5 ± 33.2	22.9 ± 6.8
	BSK II-S	2	74.0 ± 4.2	174.5 ± 2.1	18.6 ± 1.0
6	BSK II-HF	1	63.0 ± 0.0	125.0 ± 0.0	23.8 ± 0.0
	BSK II-F	1	14.0 ± 0.0	37.0 ± 0.0	16.8 ± 0.0

In this study, the PDT calculations were based on 100% survival by not counting non-motile spirochetes and excluding cultures that exhibited a preponderance of nonmotile spirochetes. During counting observations, any presence of nonmotile spirochetes was characterized. If several nonmotile spirochetes were observed and the derived PDT's were extremely prolonged, then the culture was considered dying and therefore excluded. About 40 cultures were rejected due to indications of culture death after just 24 to 48 hours of incubation. There was no consistent correlation between dying cultures and either isolate source or medium type.

Aggregation of B. burgdorferi

The aggregation or clumping of B. burgdorferi cultured in vitro has been widely observed (Aberer and Duray 1991, Barbour 1984 and 1986, Berger et al. 1985, Ewing et al. 1994, Johnson, R. 1990,

Nadelman et al. 1990, and Preac-Mursic et al. 1991). The presence of spirochete aggregates has not been described in cocultivation systems. The tendency of freshly isolated spirochetes to aggregate was lost after several passages. Aggregation may be a more natural state for this organism or a mechanism to avoid phagocytosis. (Aberer and Duray 1991, Barbour 1984, Berger et al. 1985, Johnson, R. et al. 1990, Nadelman et al. 1990) Aggregation may facilitate plasmid transfer and help conserve plasmids among the population. Although lab adapted strains lose plasmids, pathogenicity, and the tendency to form aggregates, a correlation between pathogenicity and aggregation has not been determined.

Spirochete aggregation made counting difficult. About 30 cultures were too aggregated to derive the PDT. The morphology of aggregates suggested a progression from small- to large-sized aggregate complexes. A relatively few intertwined spirochetes constituted a "spindle", then a "bundle" as more spirochetes became entwined especially at the center of the aggregate. Aggregation progressed to a "colony" with a dense spirochete center. Eventually a whitish macroscopic-sized mass of spirochetes formed on the bottom of nearly all the culture tubes. The "macrodeposit" aggregate could not be dispersed evenly by vortexing or by repeated aspiration with a pipet. Spirochete deposits in the bottom of culture tubes have been observed by other researchers culturing low passage isolates (Barbour 1983 and 1984, Burgdorfer 1985, Ewing 1994). Aberer and Duray (1991) described a variety of stained, individual B. burgdorferi morphologies and a spirochete aggregate called a "culture colony form". According to the authors, the colony looked like "spaghetti and meatballs," "curds and wheys," or "Medusa-like."

The formation of aggregates sequestered individual, countable spirochetes which artificially lengthened the PDT. Once aggregated, the spirochetes stayed aggregated. Aggregation was not simply the result of a dense spirochete concentration or length of time in culture. Aggregation may be enhanced in the presence of certain ingredients. The formation of medium- to large-sized colonies and various-sized bundles was accelerated in BSK II-S and BSK II-HS containing the human serum substitute. Spirochetes were rarely

aggregated in observations of BSK II-F cultures that contained fetal bovine serum. This was contrary to earlier observations by Dr. Stoenner (1974) in which he noted increased spirochete aggregation in medium supplemented with fetal calf serum. The BSK II-HF cultures that contained both fetal bovine serum and human albumin had little aggregation. The BSK II-H and BSK II-H50 cultures that contained human albumin produced more aggregates than the BSK II medium that contained bovine albumin.

There were instances in which the calculated PDT was faster in an interval that contained several aggregates than in a previous interval that was free of aggregates. This was unexpected. Spirochetes bound in aggregates did not disassociate. The rate of increase of countable spirochetes should be slowed as more free swimming spirochetes become bound. Consequently the PDT's would be falsely elevated and increase the PDT value. But this was not always the case. Three sets of observations listed in Table 23 show that although the number of aggregates increased, the number of countable spirochetes also increased which yielded a lower PDT.

Table 23: Unusual noncorrelation of aggregation and concentration

	t_1	t_2			t_3		
tube id	count	count	aggregates	PDT_2	count	aggregates	PDT_3
BSK II #50	8	21	sm. colony	17.2	84	more colonies	12.8
BSK II-H #43	9	23	med. colony	18.8	68	more colonies	14.7
BSK II-S #105	35	53	sm. bundles	23.4	165	more bundles	19.7

The aggregation of the spirochetes eventually prohibited this method of measuring spirochete concentration based on an even distribution of countable spirochetes suspended in the medium. Possible alternate techniques of measuring spirochete concentration for the determination of generation time are: automated cell counting, counting spirochetes coverslipped in a known low volume of culture (Berger et al. 1985), fixing the spirochetes to a slide and then staining, or measuring dry weights. Uptake of a labeled ligand

has also been used to measure growth rate (Pavia and Bittker 1988). Aggregates would confound some of these alternate methods. Addition of Tween-20 may reduce the formation of aggregates. However, spirochetes have been observed to be more susceptible to detergents and physical manipulations than other Gram negative bacteria. (Barbour and Hayes 1986, Cox et al. 1992, Holt 1978)

The occurrence of spirochete aggregates may not be crucial to evaluating the ability of a medium to support the growth of B. burgdorferi. While the formation of aggregates has decreased as the spirochetes become culture adapted, the purpose and importance of aggregation has not been determined (Aberer and Duray 1991, Barbour 1984 and 1986, Berger et al. 1985, Ewing et al. 1994, Johnson, R. et al. 1990, Nadelman et al. 1990, and Preac-Mursic et al. 1991). Comparing the concentration of spirochetes between media inoculated in parallel was subject to inoculation error. But inoculation error was minimized by increasing the number of test replicates. Determining the average population doubling times provided a more universal method of evaluating medium performance and summarizing the results from different experimental designs.

CONCLUSIONS

Dr. Barbour (1984) cited three criteria for a satisfactory culture medium for Borrelia burgdorferi. First, the medium must stimulate a population doubling time (PDT) of less than 12 hours during the log phase of growth. Second, the medium should be able to support a final density of at least 10^8 spirochetes per milliliter. Third, the medium must be able to establish a high density of spirochetes from an inoculum containing as few as one to five spirochetes. The ability of the medium to produce detectable growth from an inoculum containing very few spirochetes is important for the diagnosis and treatment of Lyme disease (Callister et al. 1990). Although the first criterion of a useful medium is proliferation of B. burgdorferi, comparing population doubling times should not be the only means of evaluating medium performance. The ability to serve as a transport medium and cryopreservative also are important. Adverse side effects of in vitro cultivation are critical evaluation tools. A reduced tendency to aggregate, to produce antigenic and other morphological changes such as the appearance of membrane blebs, the loss of plasmids, and decreased infectivity to rodents have resulted from continual in vitro culturing of B. burgdorferi.

In this study, the three criteria of a satisfactory medium were met by BSK II-H medium which contained human albumin instead of bovine albumin. Achievement of the first criterion was clearly evident. The average PDT's in fresh BSK II-H medium was less than 12 hours in cultures started from thawed stock and in two of three active passage experiments. The third active passage experiment, conducted with media that had aged two weeks, produced an average PDT in BSK II-H of 13.5 ± 1.0 hours. Fulfillment of the second criterion was not precisely measurable due to the difficulties distinguishing individual spirochetes at concentrations exceeding 10^7 spirochetes per mL and the sequestration of individual

spirochetes in the formation of aggregates. Since the spirochetes continued to be actively motile beyond the concentration of 10^7 spirochetes per mL and eventually formed macrodeposits in the bottom of the culture tubes, the concentration of spirochetes most likely exceeded 10^8 spirochetes per mL. The third criterion was resolved by observing growth from low concentration inocula. Growth of spirochetes from inocula containing approximately three spirochetes produced a higher density of spirochetes in BSK II-H than in BSK II. The substitution of human serum may have increased spirochete aggregation but decreased culture survival.

Media that contained either human or fetal bovine serum prolonged the PDT's to greater than 12 hours. When comparing the two albumin components in combination with the serum substitutions, the presence of human albumin supported spirochete growth better than did bovine albumin. The difficulties experienced with manually enumerating spirochetes and the dependence on determining PDT's to measure spirochete production indicated that additional methods of comparison are necessary to evaluate medium performance. Possible additional testing could include blind culturing studies, molecular or immunological characterization of the stored isolates, and recovery of the isolates from long term freezer storage examining not only which medium was the best cryopreservative but also which medium best supported the thawed isolates. The most significant result of this study was that in all parallel testing, BSK II-H medium containing human albumin performed as well or better than the unmodified BSK II medium containing bovine albumin.

LIST OF REFERENCES

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Aberer, E., C. Brunner, G. Suchanek, H. Klade, A. Barbour, G. Stanek, and H. Lassmann. 1989. Molecular mimicry and Lyme borreliosis. Ann. Neurol. 26:732-737.

Aberer, E. and P. H. Duray. 1991. Morphology of Borrelia burgdorferi: structural patterns of cultured Borreliae in relation to staining methods. J. Clin. Microbiol. 29(4):764-772.

Adam, T., G. S. Gassmann, C. Rasiah, and U. B. Göbel. 1991. Phenotypic and genotypic analysis of Borrelia burgdorferi isolates from various sources. Infect. Immun. 59(8):2579-2585.

Agger, W., K. L. Case, G. L. Bryant, and S. M. Callister. 1991. Lyme disease: clinical features, classifications, and epidemiology in the upper midwest. Medicine. 70(2):83-90.

Aguero-Rosenfeld, M. E., J. Nowakowski, D. F. McKenna, C. A. Carbonaro, and G. P. Wormser. 1993. Serodiagnosis in early Lyme disease. J. Clin. Microbiol. 31:3090-3095.

Anderson, J. F. 1988. Mammalian and avian reservoirs for Borrelia burgdorferi. Ann. N. Y. Acad. Sci. 539:180-191.

Anderson, J. F. 1989a. Ecology of Lyme disease. Conn. Med. 53:343-346.

Anderson, J. F. 1989b. Epizootiology of Borrelia in Ixodes tick vectors and reservoir hosts. Rev. Infect. Dis. 11 (suppl. 6): S1451-S1459.

Anderson, J. F. 1989c. Isolation and cryopreservation of the Lyme disease spirochete Borrelia burgdorferi. USFCC Newsletter 19(3):1-4.

Anderson, J. F. 1989d. Preventing Lyme disease. Rheum. Dis. Clin. North Am. 15:757-766.

Anderson, J. F., S. W. Barthold, and L. A. Magnarelli. 1990a. Infectious but nonpathogenic isolate of Borrelia burgdorferi. J. Clin. Microbiol. 28(12):2693-2699.

Anderson, J. F., R. C. Johnson, and L. A. Magnarelli. 1987. Seasonal prevalence of Borrelia burgdorferi in natural populations of white-footed mice, Peromyscus leucopus. J. Clin. Microbiol. 25(8):1564-1566.

Anderson, J. F., R. C. Johnson, L. A. Magnarelli, and F. W. Hyde. 1985. Identification of endemic foci of Lyme disease: isolation of Borrelia burgdorferi from feral rodents and ticks (Dermacentor variabilis). J. Clin. Microbiol. 22:36-38.

Anderson, J. F., R. C. Johnson, L. A. Magnarelli, and F. W. Hyde. 1986a. Culturing Borrelia burgdorferi from spleen and kidney tissues of wild-caught white-footed mice, Peromyscus leucopus. Zbl. Bakt. Hyg. A 263:34-39.

Anderson, J. F., R. C. Johnson, L. A. Magnarelli, and F. W. Hyde. 1986b. Involvement of birds in the epidemiology of the Lyme disease agent Borrelia burgdorferi. Infect. Immun. 51(2):394-396.

Anderson, J. F., and L, A, Magnarelli. 1984. Avian and mammalian hosts for spirochete-infected ticks and insects in a Lyme disease focus in Connecticut. Yale J. Biol. Med. 57:621-641.

Anderson, J. F., and L, A, Magnarelli. 1992. Epizootiology of Lyme disease and methods of cultivating Borrelia burgdorferi. Ann. N. Y. Acad. Sci. 653:52-63.

Anderson, J. F., L. A. Magnarelli, R. B. LeFebvre, and T. G. Andreadis, J. B. McAninch, G. C. Perng, and R. C. Johnson. 1989. Antigenically variable Borrelia burgdorferi isolated from cottontail rabbits and Ixodes dentatus in rural and urban areas. J. Clin. Microbiol. 27:13-20.

Anderson, J. F., L. A. Magnarelli, and K. C. Stafford III. 1990b. Bird-feeding ticks transstadially transmit Borrelia burgdorferi that infect Syrian hamsters. J. Wildl. Dis. 26:1-10.

Anthonissen, F. M., M. De Kesel, P. P. Hoet, and G. H. Bigaignon. 1994. Evidence for the involvement of different genospecies of Borrelia in the clinical outcome of Lyme disease in Belgium. Res. Microbiol. 145:327-331.

Appel, M. J. G., S. Allan, R. H. Jacobson, T. L. Lauderdale, Y. F. Chang, S. J. Shin, J. W. Thomford, R. J. Todhunter, and B. A. Summers. 1993. Experimental Lyme disease in dogs produces arthritis and persistent infection. J. Infect. Dis. 167:651-664.

Aristowsky, W. and R. Hoeltzer. 1925. Ein neuer nahrboden zur kultivierung der Spirochaeta obermeieri. Zbl. Bakt. I. Abt. Orig. 94:448-452.

Åsbrink, E. and A. Hovmark. 1985. Successful cultivation of spirochetes from skin lesions of patients with erythema chronicum migrans afzelius and acrodermatitis chronica atrophicans. Acta Path. Microbiol. Immunol. Scand. Sect. B 93:161-163.

Assous, M. V., D. Postic, P. Nevot, and G. Baranton. 1994. Individualization of two new genomic groups among American Borrelia burgdorferi sensu lato strains. FEMS Microbiol. Lett. 121(1):93-98.

Assous, M. V., D. Postic, G. Paul, P. Nevot, and G. Baranton. 1993. Western blot analysis of sera from Lyme borreliosis patients according to the genomic species of the Borrelia strains used as antigens. Eur. J. Clin. Microbiol. Infect. Dis. 12:261-268.

Austin, F. E. 1993. Maintenance of infective Borrelia burgdorferi Sh-2-82 in 4% oxygen - 5% carbon dioxide in vitro. Can. J. Microbiol. 39(12):1103-1110.

Backenson, P. B., J. L. Coleman, and J. L. Benach. 1995. Borrelia burgdorferi shows specificity of binding to glycosphingolipids. Infect. Immun. 63(8):2811-2817.

Badon, S. J., R. D. Fister, and R. G. Cable. 1989. Survival of Borrelia burgdorferi in blood products. Transfusion. 29(7):581-583.

Baranton G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. D. Grimont. 1992. Delineation of Borrelia burgdorferi sensu stricto, Borrelia garinii sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. 42:378-383.

Barbour, A. G. 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. 57:521-525.

Barbour, A. G. 1986. Cultivation of Borrelia: A historical overview. Zbl. Bakt. Hyg. A 263:11-14.

Barbour, A. G. 1988a. Laboratory aspects of Lyme borreliosis. Clin. Microbiol. Rev. 1(4):399-414.

Barbour, A. G. 1988b. Plasmid analysis of Borrelia burgdorferi, the Lyme disease agent. J. Clin. Microbiol. 26:475-478.

Barbour, A. G. 1989a. The diagnosis of Lyme disease: Rewards and perils. Ann. Intern. Med. 110(7):501-502.

Barbour, A. G. 1989b. The molecular biology of Borrelia. Rev. Infect. Dis. (suppl. 6) 11:S1470-S1474.

Barbour, A. G., W. Burgdorfer, S. F. Hayes, O. Peter, and A. Aeschlimann. 1983. Isolation of a cultivable spirochete from Ixodes ricinus ticks of Switzerland. Curr. Microbiol. 8:123-126.

Barbour, A. G., and C. F. Garon. 1988. The genes encoding major surface proteins of Borrelia burgdorferi are located on a plasmid. Ann. N. Y. Acad. Sci. 539:144-153.

Barbour, A. G. and S. F. Hayes. 1986. Biology of Borrelia species. Microbiol. Rev. 50(4):381-400.

Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier. 1986. A Borrelia-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. 52:549-554.

Barbour, A. G., R. A. Heiland, and T. R. Howe. 1985. Heterogeneity of major protein in Lyme disease borreliae: a molecular analysis of North American and European isolates. J. Infect. Dis. 152(3):478-484.

Barbour, A. G., and M. E. Schrumpf. 1986. Polymorphisms of major surface proteins of Borrelia burgdorferi. Zbl. Bakt. Hyg. A 263:83-91.

Barthold, S. W. 1991. Infectivity of Borrelia burgdorferi relative to route of inoculation and genotype in laboratory mice. J. Infect. Dis. 163:419-420.

Barthold, S. W., D. S. Beck, G. M. Hansen, G. A. Terwilliger, and K. D. Moody. 1990. Lyme borreliosis in selected strains and ages of laboratory mice. J. Infect. Dis. 162:133-138.

Barthold, S. W., D. H. Persing, A. L. Armstrong, and R. A. Peeples. 1991. Kinetics of Borrelia burgdorferi dissemination and evolution of disease after intradermal inoculation of mice. Am. J. Pathol. 139(2):263-273.

Beck, G., J. L. Benach, and G. S. Habicht. 1990. Isolation, preliminary chemical characterization, and biological activity of Borrelia burgdorferi peptidoglycan. Biochem. Biophys. Res. Commun. 167(1):89-95.

Beck, G., G. S. Habicht, J. C. Benach, and J. L. Coleman. 1985. Chemical and biologic characterization of a lipopolysaccharide extracted from the Lyme disease spirochete (Borrelia burgdorferi). J. Infect. Dis. 152:108-117.

Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. 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. New Eng. J. Med. 308:740-742.

Benach, J. L., J. L. Coleman, J. C. Garcia-Monco, and P. C.Deponte. 1988. Biological activity of Borrelia burgdorferi antigens. Ann. N. Y. Acad. Sci. 539:115-125.

Benach, J. L., J. L. Coleman, R. A. Skinner, and E. M. Baker. 1987. Adult Ixodes dammini on rabbits: A hypothesis of the development and transmission of Borrelia burgdorferi. J. Infect. Dis. 155:1300-1306.

Berg, D., K. G. Abson, and N. S. Prose. 1991. The laboratory diagnosis of Lyme disease. Arch. Dermatol. 127:866-870.

Berg, H. C. and L. Turner. 1979. Movement of microorganisms in viscous environments. Nature (London) 178:349-351.

Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman. 1992. Cultivation of Borrelia burgdorferi from erythema migrans lesions and perilesional skin. J. Clin. Microbiol. 30(2):359-361.

Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman. 1994. Cultivation of Borrelia burgdorferi from the blood of two patients with erythema migrans lesions lacking extracutaneous signs and symptoms of Lyme disease. J. Am. Acad. Dermatol. 30(1):48-51.

Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman. 1995. Cultivation of Borrelia burgdorferi from human tick bite sites: a guide to the risk of infection. J. Am. Acad. Dermatol. 32(2):184-187.

Berger, B. W., M. H. Kaplan, I. R. Rothenberg, and A. G. Barbour. 1985. Isolation and characterization of the Lyme disease spirochete from the skin of patients with erythema chronicum migrans. J. Am. Acad. Derm. 13(3):444-449.

Bergström, S., A. G. Barbour, C. F. Garon, P. Hindersson, I. S. Girons, and T. G. Schwann. 1991. Genetics of Borrelia burgdorferi. Scand J. Infect Dis. Suppl. 77:102-107.

Bergström, S., V. G. Bundoc, and A. G. Barbour. 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochaete Borrelia burgdorferi. Mol. Microbiol. 3(4):479-486.

Bisset, M. L. and W. Hill. 1987. Characterization of Borrelia burgdorferi strains isolated from Ixodid pacificus ticks in California. J. Clin. Microbiol. 25(12):2296-2301.

Boerlin, P., O. Peter, A. G. Bretz, D. Postic, G. Baranton, and J. C. Piffaretti. 1992. Population genetic analysis of Borrelia burgdorferi isolates by multilocus enzyme electrophoresis. Infect. Immun. 60(4):1677-1683.

Bohls, S. W. , J. V. Irons, and T. DeShazo. 1940. Cultivation of relapsing fever spirochetes in embryonic chick. Proc. Soc. Exp. Biol. Med. 45:375-377.

Bosler, E. M., D. P. Cohen, T. L. Schulze, C. Olsen, W. Bernard, and B. Lissman. 1988. Host responses to Borrelia burgdorferi in dogs and horses. Ann. N. Y. Acad. Sci. 539:221-234.

Bosler, E. M., R. D. Evans, and E. M. Schneider. 1992. Biopsy of canine ear tissue for detection of Borrelia burgdorferi following Ixodes dammini feeding. FEMS Microbiol. Let. 99:317-320.

Bosler, E. M., B. G. Ormiston, J. L. Coleman, J. P. Hanrahan, and J. L. Benach. 1984. Prevalence of the Lyme disease spirochete in populations of white-tailed deer and white-footed mice. Yale J. Biol. Med. 57:651-659.

Bosler, E. M. and J. L. Schulze. 1986. The prevalence and significance of Borrelia burgdorferi in the urine of feral reservoir hosts. Zbl. Bakt. Hyg. A 263:40-44.

Brunet, L. R., C. Sellitto, A. Spielman, and S. R. Telford III. 1995. Antibody response of the mouse reservoir of Borrelia burgdorferi in nature. Infect. Immun. 63:3030-3036.

Bruynoghe, R. 1928. A contribution to the study of relapsing fever. J. State Med. 36:3-20.

Bunkis, J., B. Olsen, G. Westman, and S. Bergström. 1995. Variable serum immunoglobulin responses against different Borrelia burgdorferi sensu lato species in a population at risk for and patients with Lyme disease. J. Clin. Microbiol. 33(6):1473-1478.

Burgdorfer, W. 1984. Discovery of the Lyme disease spirochete and its relation to tick vectors. Yale J. Biol. Med. 57:515-520.

Burgdorfer, W. 1989. Vector/host relationships of the Lyme disease spirochete, Borrelia burgdorferi. Rheum. Dis. Clin. North Am. 15:775-787.

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.

Burgdorfer, W. and K. L. Gage. 1986. Susceptibility of the black-legged tick, Ixodes scapularis to the Lyme disease spirochete, Borrelia burgdorferi. Zbl. Bakt. Hyg. A 263:15-20.

Burgdorfer, W. and K. L. Gage. 1987. Susceptibility of the hispid cotton rat (Sigmodon hispidus) to the Lyme disease spirochete (Borrelia burgdorferi). Am. J. Trop. Med. Hyg. 35:355-359.

Burgdorfer, W., S. F. Hayes, and J. L. Benach. 1988. Development of Borrelia burgdorferi in Ixodid tick vectors. Ann. N. Y. Acad. Sci. 539:172-179.

Burgdorfer, W., S. F. Hayes, and D. Corwin. 1989. Pathophysiology of the Lyme disease spirochete, Borrelia burgdorferi, in ixodid ticks. Rev. Infect. Dis. 11:S1442-1450.

Burgdorfer, W., R. S. Lane, A. G. Barbour, R. A. Gresbrink, and J. R. Johnson. 1985. The western black-legged tick, Ixodes pacificus: a vector of Borrelia burgdorferi. Am. J. Trop. Med. Hyg. 34:925-930.

Burgdorfer, W. and T. G. Schwan. 1991. Lyme borreliosis: a relapsing fever-like disease? Scand. J. Infect. Dis. Suppl. 77:17-22.

Burgess, E. C. 1988. Borrelia burgdorferi infection in Wisconsin horses and cows. Ann. N. Y. Acad. Sci. 539:235-243.

Burgess, E. C., T. E. Amundson, J. P. Davis, R. A. Kaslow, and R. Edelman. 1986. Experimental inoculation of Peromyscus spp. with Borrelia burgdorferi: evidence of contact transmission. Am J. Trop. Med. Hyg. 35:355-359.

Burgess, E. C., J. B. French Jr., and A Gendron-Fitzpatrick. 1990. Systemic disease in Peromyscus leucopus associated with Borrelia burgdorferi infection. Am J. Trop. Med. Hyg. 42(3):254-259.

Burgess, E. C., and L. A. Windberg. 1989. Borrelia sp. infection in coyotes, black-tailed jack rabbits, and desert cottontails in southern Texas. J. Wildl. Dis. 25:47-51.

Burkot, T. R., J. Piesman, and R. A. Wirtz. 1994. Quantitation of the Borrelia burgdorferi outer surface protein A in Ixodes scapularis: fluctuations during tick life cycle, doubling times, and loss while feeding. J. Infect. Dis. 170:883-889.

Callister, S. M., W. A. Agger, R. F. Schell, and K. M. Brand. 1989. Efficacy of the urinary bladder for isolation of Borrelia burgdorferi from naturally infected, wild Peromyscus leucopus. J. Clin. Microbiol. 27(4):773-774.

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(2):363-365.

Callister, S. M., R. F. Schell, S. D. Lovrich, and D. A. Jobe. 1994. Lyme disease: laboratory diagnosis and serologic testing. Endeavour 18(2):80-84.

Campbell, G. L., W. S. Paul, M. E. Schriefer, R. B. Craven, K. E. Robbins, and D. T. Dennis. 1995. Epidemiologic and diagnostic studies of patients with suspected early Lyme disease, Missouri, 1990-1993. J. Infect. Dis. 172:470-480.

Campbell, G. L., J. Piesman, P. D. Mitchell, T. J. Quan, K. D. Reed, and D. T. Dennis. 1994. An evaluation of media for transport of tissues infected with Borrelia burgdorferi. Am. J. Clin. Pathol. 101(2):154-156.

Canica, M. M., F. Nato, L. Dumerle, J. C. Mazie, G. Baranton, and D. Postic. 1993. Monoclonal antibodies for identification of B. afzelii sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand. J. Infect. Dis. 25:441-448.

Centers for Disease Control. 1989. Lyme disease - United States, 1987 and 1988. MMWR 38:668-672.

Centers for Disease Control. 1992. Lyme disease surveillance summary. Ft. Collins, Co. 3(3)1-5.

Centers for Disease Control. 1993a. Emerging infectious diseases: Lyme disease - United States, 1991-1992. MMWR 42:345-348.

Centers for Disease Control. 1993b. Emerging infectious diseases: Lyme disease - United States, 1993. MMWR 43:564-572.

Centers for Disease Control. 1995. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR 44(31):590-591.

Chu, H. J., L. G. Chavez, B. M Blumer, R. W. Sebring, T. L. Wasmoen, and W. M. Acree. 1992. Immunogenicity and efficacy study of a commercial Borrelia burgdorferi bacterin. JAVMA 201(3):403-418.

Ciesielski, C. A., L. E. Markowitz, R. Horsley, A. W. Hightower, H. Russell, and C. V. Broome. 1989. Lyme disease surveillance in the United States, 1983-1986. Rev. Infect. Dis. 11:1435-1441.

Cinco, M., E. Banfi, D. Balanzin, C. Godeas, and E. Panfili. 1991. Evidence for (lipo)oligosaccharides in Borrelia burgdorferi and their serological specificity. Fems Microbiol. Immunol. 76(1):33-38.

Cluss, R. G. and J. T. Boothby. 1990. Thermoregulation of protein synthesis in Borrelia burgdorferi. Infect. Immun. 58(4): 1038-1042.

Cohen, R. 1991. We think it's Lyme. Am. J. Med. 90:273-274.

Coleman, J. L. and J. L. Benach. 1987. Isolation of antigenic components from the Lyme disease spirochete: their role in early activity of skin and joints. J. Infect. Dis. 155:756-765.

Coleman, J. L. and J. L. Benach. 1992. Characterization of antigenic determinants of Borrelia burgdorferi shared by other bacteria. J. Infect. Dis. 165:658-666.

Coleman, J. L., J. L. Benach, G. Beck, and G. S. Habicht. 1986. Isolation of the outer envelope from Borrelia burgdorferi. Zbl. Bakt. Hyg. A 263:123-126.

Coleman, J. L., T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach. 1995. Borrelia burgdorferi binds plasminogen, resulting in enhanced penetration of enothelial monolayers. Infect. Immun. 63(7):2478-2484.

Comstock, L. E., E. Fikrig, J. Shoberg, R. A. Flavell, and D. D. Thomas. 1993. A monoclonal antibody to OspA inhibits association of Borrelia burgdorferi with human endothelial cells. Infect. Immun. 61:423-431.

Comstock, L. E., and D. D. Thomas. 1989. Penetration of endothelial cell monolayers by Borrelia burgdorferi. Infect. Immun. 57:1626-1628.

Cox, D. L., P. Chang, A. McDoweall, and J. D. Radolf. 1992. The outer membrane, not a coat of host proteins, limits the antigenicity of virulent Treponema pallidum. Infect. Immun. 60:1076-1083.

Craft, J. E., D. K. Fischer, G. T. Shimamato, and A. C. Steere. 1986. Antigens of Borrelia burgdorferi recognized during Lyme disease: Appearence of a new immunoglobulin M response and expansion of the immunoglobulin G response late in the illness. J. Clin. Invest. 78:934-939.

Cutler, S. J. and D. J. M. Wright. 1989. Comparison of immunofluorescence and enzyme-linked immunosorbent assays for diagnosing Lyme disease. J. Clin. Pathol. 42:869-871.

Daniels, T. J., D. Fish, J. F. Levine, M. A. Greco, A. T. Eaton, P. J. Padgett, and D. A. Lapointe. 1993. Canine exposure to Borrelia burgdorferi and prevalence of Ixodes dammini (Acari: Ixodidae) on deer as a measure of Lyme disease risk in the northeastern United States. J. Med. Entomol. 30 (1):171-178.

Dattwyler, R. J., J. J. Halperin, H. Pass, and B. J. Luft. 1987. Ceftriaxone as effective therapy in refractory Lyme disease. J. Infect. Dis. 155:1322-1325.

Dattwyler, R. J. and J. A. Thomas. 1986. Cellular immune response in Lyme disease: The response to mitogens, live Borrelia burgdorferi, NK cell function and lymphocytic subsets. Zbl. Bakt. Hyg. A 263:151-159.

Dattwyler, R. J., D. J. Volkman, J. J. Halperin, B. J. Luft, J. Thomas, and M. G. Golightly. 1988. Specific immune responses in Lyme borreliosis: Characterization of T cell and B cell responses to Borrelia burgdorferi. Ann N. Y. Acad. Sci. 539:93-102.

Dattwyler, R. J., D. J. Volkman, and B. J. Luft. 1989. Immunologic aspects of Lyme borreliosis. Rev. Infect. Dis. 11(suppl. 6):S1494-1498.

Debue, M., P. Gautier, C. Hackel, A. Van Elsen, A. Herzog, G. Bigaignon, and A. Bollen. 1991. Detection of Borrelia burgdorferi in biological samples using the polymerase chain reaction assay. Res. Microbiol. 142:565-572.

Demaerschalck, I., A. Ben Messaoud, M. De Kesel, B. Hoyois, Y. Lobet, P. Hoet, G. Bigaignon, A. Bollen, and E. Godfroid. 1995. Simultaneous presence of different Borrelia burgdorferi genospecies in biological fluids of Lyme disease patients. J. Clin. Microbiol. 33(3):602-608.

Dever, L. L., J. H. Jorgensen, and A. G. Barbour. 1992. In vitro antimicrobial susceptibility testing of Borrelia burgdorferi: a microdilution MIC method and time-kill studies. J. Clin. Microbiol. 30(10):2692-2697.

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.

Dorward, D. W., T. G. Schwan, and C. F. Garon. 1991. Immune capture detection of Borrelia burgdorferi antigens in urine, blood, or tissues from infected ticks, mice, dogs, and hamsters. J. Clin. Microbiol. 29:1162-1170.

Dressler, F., J. A. Whalen, B. N. Reinhardt, and A. C. Steere. 1993. Western blotting in the serodiagnosis of Lyme disease. J. Infect. Dis. 167(February):392-400.

Dressler, F., N. H. Yoshinari, and A. C. Steere. 1991. The T-cell proliferative assay in the diagnosis of Lyme disease. Ann. Intern. Med. 115:533-539.

Drummond, R. O., T. M. Whetstone, and J. A. Miller. 1981. Control of ticks systemically with Merck MK-933, an avermectin. J. Econ. Entomol. 74:432-436.

Duffy, D. C., S. R. Campbell, D. Clark, C. DiMotta, and S. Gurney. 1994a. Ixodes scapularis (Acari: Ixodidae) deer ticks mesoscale populations in natural areas: effects of deer, area, and location. J. Med. Entomol. 31(1):152-158.

Duffy, D. C., D. D. Clark, S. R. Campbell, S. Gurney, R. Perello, and N. Simon. 1994b. Landscape patterns of abundance of Ixodes scapularis (Acari:Ixodidae) on Shelter Island, New York. J. Med. Entomol. 31(6):875-879.

Duffy, D. C., R. Downer, and C. Brinkley. 1992. The effectiveness of helmeted guineafowl in the control of the deer tick, the vector of Lyme disease. Wilson Bull. 104(2):342-345.

Duray, P. H. 1989a. Clinical pathologic correlations of Lyme disease. Rev. Infect. Dis. 11 (suppl. 6):S1487-S1493.

Duray, P. H. 1989b. Histopathology of clinical phases of human Lyme disease. Rheum. Dis. Clin. North Am. 15:691-710.

Duray, P. H. and A. C. Steere. 1988. Clinical pathological correlations of Lyme disease by stage. Ann N. Y. Acad. Sci. 539:65-79.

Eberson, F. and W. G. Mossmann. 1931. Spirocheta hispanicum. Application in fever therapy and diseases on central nervous system. Proc. Soc. Exp. Biol. Med. 29:108-111.

Engstrom, S. M., E. Shoop, R. C. Johnson. 1995. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J. Clin. Microbiol. 33(2):419-427.

Ewing, C., A. Scorpio, D. R. Nelson, and T. N. Mather. 1994. Isolation of Borrelia burgdorferi from saliva of the tick vector, Ixodes scapularis. J. Clin. Microbiol. 32(3):755-758.

Falco, R. C., H. A. Smith, D. Fish, B. A. Mojica, M. A. Bellinger, H. L. Harris, and K. E. Hechemy. 1993. The distribution of canine exposure to Borrelia burgdorferi in a Lyme disease endemic area. Am. J. Public Health 83(9):1305-1310.

Fawcett, P. T., A. E. O'Brien, and R. A. Doughty. 1989. An adsorption procedure to increase the specificity of enzyme-linked immunosorbent assays for Lyme disease without decreasing sensitivity. Arthritis Rheum. 32:1041-1044.

Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell. 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. Science. 250(4980):553-556.

Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell. 1992a. Long-term protection of mice from Lyme disease by vaccination with Osp A. Infect. Immun. 60(3):773-777.

Fikrig, E., S. R. Telford III, S. W. Barthold, F. S. Kantor, A. Spielman, and R. A. Flavell. 1992b. Elimination of Borrelia burgdorferi from vector ticks feeding on OspA-immunized mice. Proc. Natl. Acad. Sci. USA 89:5418

Fikrig, E., S. R. Telford III, R. Wallich, M. Chen, Y. Lobert, F. R. Matuschka, R. B. Kimsey, F. S. Kantor, S. W. Barthold, A. Spielman, and R. A. Flavell. 1995. Vaccination against Lyme disease caused by diverse Borrelia burgdorferi. J. Exp. Med. 181(1):215-221.

Fish, D. and T. J. Daniels. 1990. The role of medium-sized mammals as reservoirs of Borrelia burgdorferi in southern New York. J. Wildl. Dis. 26:339-345.

Fridriksdottir, V., L. L. Nesse, and R. Gudding. 1992. Seroepidemiological studies of Borrelia burgdorferi infection in sheep in Norway. J. Clin. Microbiol. 30(5):1271-1277.

Fumarola, D., I. Munno, C. Marcuccio, and G. Miragliotta. 1986. Endotoxin-like activity associated with Lyme disease Borreliae. Zbl. Bakt. Hyg. A 263:142-145.

Fung, B. P., G. L. McHugh, J. M. Leong, and A. C. Steere. 1994. Humoral immune response to outer surface protein C of Borrelia burgdorferi in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection. Infect. Immun. 62:3213-3221.

Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, M. J. Waring. 1981a. Antibiotic inhibitors of ribosome function, ch. 6. *In* The Molecular Basis of Antibiotic Action, 2nd edition. John Wiley and Sons. London.

Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, M. J. Waring. 1981b. Biochemical targets for drug action, ch. 2. *In* The Molecular Basis of Antibiotic Action, 2nd edition. John Wiley and Sons. London.

Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, M. J. Waring. 1981c. Inhibitors of nucleic acid synthesis, ch. 5. *In* The Molecular Basis of Antibiotic Action, 2nd edition. John Wiley and Sons. London.

Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, M. J. Waring. 1981d. Inhibitors of bacterial and fungal cell wall synthesis, ch. 3. *In* The Molecular Basis of Antibiotic Action, 2nd edition. John Wiley and Sons. London.

Garci-Monco, J. C., B. Fernandez-Villar, and J. L. Benach. 1989. Adherence of the Lyme disease spirochetes to glial cells and cells of glial origin. J. Infect. Dis. 160:497-506.

Garon, C. F., D. W. Dorward, and M. D. Corwin, 1989. Structural features of Borrelia burgdorferi - the Lyme disease spirochete: silver staining for nuceic acids. Scanning Microsc. Suppl. 3:109-115.

Georgilis, K., A. C. Steere, and M. S. Klempner. 1991. Infectivity of Borrelia burgdorferi correlates with resistance to elimination by phagocytic cells. J. Infect. Dis. 163:150-155.

Gern, L., U. E. Schaible, and M. M. Simon. 1993. Mode of inoculation of the Lyme disease agent Borrelia burgdorferi influences infection and immune responses in inbred strains of mice. J. Infect. Dis. 167:971-975.

Gill, J. S., R. G. McLean, D. F. Neitzel, and R. C. Johnson. 1993. Serologic analysis of white-tailed deer sera for antibodies to Borrelia burgdorferi by enzyme-linked immunosorbent assay and Western immunoblotting. J. Clin. Microbiol. 31(2):318-322.

Golde, W. T., K. J. Kappel, G. Dequesne, C. Feron, D. Plainchamp, C. Caplau, and Y. Lobet. 1994. Tick transmission of Borrelia burgdorferi to inbred strains of mice induces an antibody response to P39 but not to outer surface protein A. Infect. Immun. 62(6):2625-2627.

Goldstein, S. F., N. W. Charon, and J. A. Kreiling. 1994. Borrelia burgdorferi swims with a planar waveform similar to that of eukaryotic flagella. Proc. Natl. Acad. Sci. USA 91(8):3433-3437.

Golightly, M. G., J. A. Thomas, D. J. Volkman, and R. J. Dattwyler. 1988. Modulation of natural killer cell activity by Borrelia burgdorferi. Ann N. Y. Acad. Sci. 539:103-111.

Goodman, J. L., P. Jurkovich, C. Kodner, and R. C. Johnson. 1991. Persistent cardiac and urinary tract infections with Borrelia burgdorferi in experimentally infected Syrian hamsters. J. Clin. Microbiol. 29(5):894-896.

Greene, R. T., R. L. Walker, E. C. Burgess, and J. F. Levine. 1988. Heterogeneity in immunoblot patterns obtained by using four strains of Borrelia burgdorferi and sera from naturally exposed dogs. J. Clin. Microbiol. 26(11):2287-2291.

Grodzicki, R. L. and A. C. Steere. 1988. Comparison of immunoblotting and indirect enzyme-linked immunosorbent assay using different antigen preparations for diagnosing early Lyme disease. J. Infect. Dis. 157(4):790-797.

Gumstorp, I. 1991. Lyme borreliosis from a patient's view-point. Scand. J. Infect. Dis. Suppl. 77:15-16.

Guner, E. S. 1994. Retention of B. burgdorferi pathogenicity and infectivity after multiple passages in a co-culture system. Experientia. 50(1):54-59.

Guy, E. C. and A. M. Turner. 1989. Seronegative neuroborreliosis. Lancet. 1(8635):441.

Guy, E. L. and G. Stanek. 1991. Detection of Borrelia burgdorferi in patients with Lyme disease by the polymerase chain reaction. J. Clin. Pathol. 44(7):610-611.

Habicht, G. S., G. Beck, J. L. Benach, and J. L. Coleman. 1986. Borrelia burgdorferi lipopolysaccharide and its role in the pathogenesis of Lyme disease. Zbl. Bakt. Hyg. A 263:137-141.

Hackman, R. H. and B. K. Filshie. 1982. The tick cuticle, p.1-42. *In* Physiology of ticks. F. D. Obenchain and R. Galum (ed.). Pergamon Press, Oxford.

Halperin, J. L. 1991. North American Lyme neuroborreliosis. Scand. J. Infect. Dis. Suppl. 77:74-80.

Hansen, K., and E. Åsbrink. 1989. Serodiagnosis of erythema migrans and acrodermatitis chronica atrophicans by the Borrelia burgdorferi flagellum enzyme-linked immunosorbent assay. J. Clin. Microbiol. 27(3):545-551.

Hansen, K., J. M. Bangsborg, H. Fjordvang, N. S. Pederson, and P Hindersson. 1988a. Immunochemical characterization of and isolation of the gene for a Borrelia burgdorferi immunodominant 60-kilodalton antigen common to a wide range of bacteria. Infect. Immun. 56:2049-2053.

Hansen, K., P. Hindersson, and N. S. Pederson. 1988b. Measurement of antibodies to the Borrelia burgdorferi flagellum improves serodiagnosis of the disease. J. Clin. Microbiol. 26:338-346.

Hansen, K., K. Pii, and A. M. Lebech. 1991. Improved immunoglobulin M serodiagnosis in Lyme borreliosis by using a mu-capture enzyme-linked immunosorbent assay with biotinylated Borrelia burgdorferi flagella. J. Clin. Microbiol. 29(1):166-173.

Hayes, S. F., W. Burgdorfer, and A. G. Barbour. 1983. Bacteriophage in the Ixodes dammini spirochete, etiological agent of Lyme disease. J. Bacteriol. 154:1436-1439.

Hechemy, K. E., H. L. Harris, M. J. Duerr, J. L. Benach, and C. B. Reimer. 1988. Immunoglobulin G (IgG) subclasses specific to Borrelia burgdorferi in patients with Lyme disease. Ann. N. Y. Acad. Sci. 539:162-169.

Herzer, P. 1991. Joint manifestations of Lyme borreliosis in Europe. Scand. J. Infect. Dis. Suppl. 77:55-63

Hofmeister, E. K. and J. E. Childs. 1995. Analysis of Borrelia burgdorferi sequentially isolated from Peromyscus leucopus captured at a Lyme disease enzootic site. J. Infect. Dis. 172(2):462-469.

Hofmeister, E. K., R. B. Markham, J. E. Childs, and R. R. Arthur. 1992. Comparison of polymerase chain reaction and culture for detection of Borrelia burgdorferi in naturally infected Peromyscus leucopus and experimentally infected C.B-17 scid/scid mice. J. Clin. Microbiol. 30(10):2625-2631.

Holt, S. C. 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. 42:114-160.

Hovind-Hougen, K., E. Åsbrink, G. Stiernstedt, A. C. Steere, and A. Hovmark. 1986. Ultrastructural differences among spirochetes isolated from patients with Lyme disease and related disorders, and from Ixodes ricinus. Zbl. Bakt. Hyg. A 263:103-111.

Howe, T. R., F. W. LaQuier, and A. G. Barbour. 1986. Organization of genes encoding two outer membrane proteins of the Lyme disease agent Borrelia burgdorferi within a single transcriptional unit. Infect. Immun. 54(1):207-212.

Hu, C. M., L. Gern, and A. Aeschlimann. 1992. Changes in the protein profile and antigenicity of different Borrelia burgdorferi strains after reintroduction to Ixodes ricinus ticks. Parasite Immunol. (Okf.) 14:415-427.

Hughes, C. A. N., C. B. Kodner, and R. C. Johnson. 1992. DNA analysis of Borrelia burgdorferi NCH-1, the first northcentral U.S. human Lyme disease isolate. J. Clin. Microbiol. 30(3):698-703.

Hyde, F. W. and R. C. Johnson. 1986. Genetic analysis of Borrelia. Zbl. Bakt. Hyg. A 263:119-122.

Hyde, F. W. and R. C. Johnson. 1988. Characterization of a circular plasmid from Borrelia burgdorferi, etiologic agent of Lyme disease. J. Clin. Microbiol. 26(10):2203-2205.

Isogai, E., H. Isogai, H. Kawabata, T. Masuzawa, Y. Yanagihara, K. Kimura, T. Sakai, Y. Azuma, N. Fujii, and S. Ohno. 1994. Lyme disease spirochetes in a wild fox (Vulpes vulpes schrencki) and in ticks. J. Wildl. Dis. 30(3):439-444.

Jaenson, T. G. T., D. Fish, H. Grinsberg, J. S. Gray, T. N. Mather, and J. Piesman. 1991. Methods for control of ticks vectors of Lyme borreliosis. Scand. J. Infect. Dis. Suppl. 77:151-157.

Jobe, D. A., S. M. Callister, L. C. Lim, S. D. Lovrich, and R. F. Schell. 1994. Ability of canine Lyme disease vaccine to protect hamsters against infection with several isolates of Borrelia burgdorferi. J. Clin. Microbiol. 32(3):618-622.

Jobe, D. A., S. M. Callister, and R. F. Schell. 1993. Recovery of Borrelia burgdorferi by filtration. J. Clin. Microbiol. 31(7):1896-1898.

Johnson, B. J. B., C. M. Happ, L. W. Mayer, and J. Piesman. 1992. Detection of Borrelia burgdorferi in ticks by species-specific amplification of the flagellin gene. Am. J. Trop. Med. Hyg. 47:730-741.

Johnson, R. C. 1981. Introduction to the spirochetes, p. 533-591. *In* The Prokaryotes: a handbook on habitats, isolation, and identification of bacteria. P. M. Starr, H. Stolp, and H. G. Truper (ed.). Springer - Verlag, New York.

Johnson, R. C. 1989. Isolation techniques for spirochetes and their sensitivity to antibiotics in vitro and in vivo. Rev. Infect. Dis. 11(suppl. 6):S1505-1510.

Johnson, R. C., F. W. Hyde, and C. M. Rumpel. 1984a. Taxonomy of the Lyme disease spirochetes. Yale J. Biol. Med. 57:529-537.

Johnson, R. C., F. W. Hyde, G. P. Schmid, and D. J. Brenner. 1984b. Borrelia burgdorferi sp. nov.: etiological agent of Lyme disease. Int. J. Syst. Bacteriol. 34:496-497.

Johnson, R. C., C. B. Kodner, P. J. Jurkovich, and J. J. Collins. 1990. Comparative in vitro and in vivo susceptibilities of the Lyme disease spirochete Borrelia burgdorferi to cefuroxime and other antimicrobial agents. Antimicrob. Agents Chemother. 34(11):2133-2136.

Johnson, R. C., C. Kodner, and M. E. Russell. 1986. Active immunization of hamsters against experimental infection with Borrelia burgdorferi. Infect. Immun. 54(3):897-898.

Johnson, R. C., C. Kodner, and M. E. Russell. 1987. In vitro and in vivo susceptibility of the Lyme disease spirochete, Borrelia burgdorferi, to four antimicrobial agents. Antimicrob. Agents Chemother. 31(2):164-167.

Johnson, R. C., C. B. Kodner, M. E. Russell, and P. H. Duray. 1988. Experimental infection of the hamster with Borrelia burgdorferi. Ann. N. Y. Acad. Sci. 539:258-263.

Johnson, S. E., G. C. Klein, G. P. Schmid, G. S. Bowen, J. C. Feeley, and T. Schulze. 1984. Lyme disease: a selective medium for isolation of the suspected etiologic agent, a spirochete. J. Clin. Microbiol. 19(1):81-82.

Johnson, S. E., B. Swaminathan, P. Moore, C. V. Broome, and M. Parvin. 1990. Borrelia burgdorferi: survival in experimentally infected human blood processed for transfusion. J. Infect. Dis. 162:557-559.

Johnston, A. M., M. Kober, and T. H. Callaway. 1992. Enzyme immunoassay with Western blot confirmation for Lyme disease testing. Lab. Med. 23(10):663-666.

Jonsson, M., L. Noppa, A. G. Barbour, and S. Bergström. 1992. Heterogeneity of outer membrane proteins in Borrelia burgdorferi: comparison of osp operons of three isolates of different geographic origins. Infect. Immun. 60(5):1845-1853.

Kaell, A. T., D. J. Volman, P. D. Gorevic, and R. J. Dattwyler. 1990. Positive Lyme serology in subacute bacterial endocarditis. JAMA 264:2916-2918.

Karlsson, M. 1990. Aspects of the diagnosis of Lyme borreliosis. Scand. J. Infect. Dis. (suppl. 67):19-48.

Karlsson, M., K. Hovind-Hougen, B. Svenungsson, and G. Stiernstedt. 1990. Cultivation and characterization of spirochetes from cerebrospinal fluid of patients with Lyme borreliosis. J. Clin. Microbiol. 28(3):473-479.

Karlsson, M., I. Mollegard, G. Stiernstedt, and B. Wretlind. 1989. Comparison of Western blot and enzyme-linked immunosorbent assay for serodiagnosis of Lyme borreliosis. Eur. J. Clin. Microbiol. Infect. Dis. 8:871-877.

Kazmierczak, J. J., T. E. Amundson, and E. C. Burgess. 1988. Borreliosis in free-ranging black bears from Wisconsin. J. Wildl. Dis. 24:366-368.

Kazmierczak, J. J. and F. E. Sorhage. 1993. Current understanding of Borrelia burgdorferi infection, with emphasis on its prevention in dogs. JAVMA 203(11):1524-1528.

Keller, D., F. T. Koster, D. H. Marks, P. Hosbach, L. F. Erdile, and J. P. Mays. 1994. Safety and immunogenicity of a recombinant outer surface protein A Lyme vaccine. JAMA 271(22):1764-1768.

Kelly, R. T. 1971. Cultivation of Borrelia hermsii. Science. 173:443-444.

Kelly, R. T. 1976. Cultivation and physiology of relapsing fever Borreliae, p. 87-94. *In* The Biology of Parasitic Spirochetes. R. C. Johnson (ed.). Academic Press, New York.

Kelly, R. T. 1984. Borrelia, vol.1, p. 57-62. *In* Bergey's Manual of Systematic Bacteriology. N. R. Krieg and J. G. Holt (ed.). Williams and Wilkins Press. Baltimore.

Kimsey, R. B. and A. Spielman. 1990. Motility of Lyme disease spirochete in fluids as viscous as the extracellular matrix. J. Infect. Dis. 162(5):1205-1208.

Kligler, J. T. and O. H. Robertson. 1922. The cultivation and biological characteristics of Spirochaeta obermeieri (Recurrentis). J. Exp. Med. 35:303-316.

Kochi, S. K., R. C. Johnson, and A. P. Dalmasso. 1991. Complement-mediated killing of the Lyme disease spirochete Borrelia burgdorferi: role of antibody in formation of an effective membrane attack complex. J. Immunol. 146(11):3964-3970.

Korenberg, E. I. 1994. Comparative ecology and epidemiology of Lyme disease and tick-borne encephalitis in the former Soviet Union. Parasit. Today 10(4):157-160.

Kornblatt, A. N., A. C. Steere, and D. G. Brownstein. 1984. Experimental Lyme disease in rabbits: spirochetes found in erythema migrans and blood. Infect. Immun. 46:220-223.

Kornblatt, A. N., P. H. Urband, and A. C. Steere. 1985. Arthritis caused by Borrelia burgdorferi in dogs. J. Am. Vet. Med. Assoc. 186:960-964.

Kristoferitsch, W. 1991. Neurological manifestations of Lyme borreliosis: clinical definition and differential diagnosis. Scand. J. Infect. Dis. Suppl. 77:64-73.

Kron, M. A., A. Gupta, and C. D. MacKenzie. 1991. Identification of related DNA sequences in Borrelia burgdorferi and two strains of Leptospira interrogans by using polymerase chain reaction. J. Clin. Microbiol. 29(10):238-2340.

Kuiper, H., A. P. van Dam, L. Spanjaard, B. M. de Jongh, A. Widjojokusumo, T. C. Ramselaar, I. Cairo, K. Vos, and J. Dankert. 1994. Isolation of Borrelia burgdorferi from biopsy specimens taken from healthy-looking skin of patients with Lyme borreliosis. J. Clin. Microbiol. 32(3):715-720.

Kurtti, T. J., U. G. Munderloh, G. G. Ahlstrand, and R. C. Johnson. 1988. Borrelia burgdorferi in tick cell culture: growth and cellular adherence. J. Med. Entomol. 25(4):256-261.

Kurtti, T. J., U. G. Munderloh, R. C. Johnson, and G. G. Ahlstrand. 1987. Colony formation and morphology in Borrelia burgdorferi. J. Clin. Microbiol. 25(11):2054-2058.

Kurtti, T. J., U. G. Munderloh, D. E. Krueger, R. C. Johnson, and T. G. Schwan. 1993. Adhesion to and invasion of cultured ticks (Acarina:Ixodidae) cells by Borrelia burgdorferi (spirochaetales:spirochaetaceae) and maintenance of infectivity. J. Med. Entomol. 30(3):586-596.

Lam, T. T., T. P. Nguyen, R. R. Montgomery, F. S. Kantor, E. Fikrig, and R. A. Flavell. 1994. Outer surface proteins E and F of Borrelia burgdorferi, the agent of Lyme disease. Infect. Immun. 62(1):290-298.

Lancaster Jr., J. L., J. S. Simco, and R. L. Kilgore. 1982. Systemic efficacy of ivermectin MK-933 against the lone star tick. J. Econ. Entomol. 75:242-244.

Lane, R. S. 1989. Treatment of clothing with a permethrin spray for personal protection against the western black-legged tick, Ixodes pacificus (Acari:Ixodidae). Exper. Appl. Acar. 6:343-352.

Lane, R. S. 1990. Infection of deer mice and piñon mice (Peromyscus spp.) with spirochetes at a focus of Lyme borreliosis in northern California, U. S. A. Bull. Soc. Vector. Ecol. 15(1):25-32.

Lane, R. S. and W. Burgdorfer. 1986. Potential role of native and exotic deer and their associated ticks (Acari: Ixodidae) in the ecology of Lyme disease in California, USA. Zbl. Bakt. Hyg. A 263:55-64.

Lane, R. S. and W. Burgdorfer. 1987. Transovarial and transstadial transmission of Borrelia burgdorferi in the western black-legged tick, Ixodes pacificus (Acari: Ixodidae). Am. J. Trop. Med. Hyg. 37(1):188-192.

Lane, R. S. and P. E. Lavoie. 1988. Lyme borreliosis in California: acarological, clinical, and epidemiological studies. Ann. N. Y. Acad. Sci. 539:192-203.

Lane, R. S. and J. A. Pascocello. 1989. Antigenic characteristics of Borrelia burgdorferi isolates from Ixodid ticks in California. J. Clin. Microbiol. 27(10):2344-2349.

Lane, R. S., J. Piesman, and W. Burgdorfer. 1991. Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe. Annu. Rev. Entomol. 36:587-609.

Lebech, A. M., O. Clemmensen, and K. Hansen. 1995. Comparison of in vitro culture, immunohistochemical staining, and PCR for detection of Borrelia burgdorferi in tissue from experimentally infected animals. J. Clin. Microbiol. 33(9):2328-2333.

Lebech, A. M., P. Hindersson, J. Vuust, and K. Hansen. 1991. Comparison of in vitro culture and polymerase chain reaction for detection of Borrelia burgdorferi in tissue from experimentally infected animals. J. Clin. Micro. 29(2):236-243.

LeFebvre, R. B., R. S. Lane, G. C. Perng, J. A. Brown, and R. C. Johnson. 1990. DNA and protein analysis of tick-derived isolates of Borrelia burgdorferi from California. J. Clin. Microbiol. 28:700-70744.

LeFebvre, R. B., G. C. Perng, and R. C. Johnson. 1989. Characterization of Borrelia burgdorferi isolates by restriction endonuclease analysis and DNA hybridization. J. Clin. Microbiol. 27(4):636-639.

Lengl-JanBen, B., A. F. Strauss, A. C. Steere, and T. Kamradt. 1994. The T helper cell response in Lyme arthritis: differential recognition of Borrelia burgdorferi outer surface protein A in patients with treatment-resistant or treatment-responsive Lyme arthritis. J. Exp. Med. 180(6):2069-2078.

Levine, J. F., M. L. Wilson, and A. Spielman. 1985. Mice as reservoirs of the Lyme disease spirochete. Am. J. Trop. Med. Hyg. 34:355-360.

Ley, C., I. H. Davila, N. M. Mayer, R. A. Murray, G. W. Rutherford, and A. L. Reingold. 1994. Lyme disease in northwestern coastal California. West. J. Med. 160(6):534-539.

Lindenmayer, J. M., D. Marshall, and A. B. Onderdonk. 1991. Dogs as sentinels for Lyme disease in Massachusetts. Am. J. Public Health 81:1448-1455.

Lindenmayer, J. M., M. Weber, J. Bryant, E. Marquez, and A. B. Onderdonk. 1990. Comparison of indirect immunofluorescent-antibody assay, enzyme-linked immunosorbent assay, and Western immunoblot for the diagnosis of Lyme disease in dogs. J. Clin. Microbiol. 28(1):92-96.

Lindsay, L. R., I. K. Barker, G. A. Surgeoner, S. A. McEwen, L. A. Elliott, and J. Kolar. 1991. Apparent incompetence of Dermacentor variabilis (Acari: Ixodidae) and fleas (Insecta: Siphonaptera) as vectors of Borrelia burgdorferi in an Ixodes dammini endemic area of Ontario, Canada. J. Med. Entomol. 28(5):750-753.

Livesley, M. A., D. Carey, L. Gern, and P. A. Nuttall. 1994. Problems of isolating Borrelia burgdorferi from ticks collected in United Kingdom foci of Lyme disease. Med. Vet. Entomol. 8:172-178.

Livesley, M. A., I. P. Thompson, L. Gern, and P. A. Nutthall. 1993. Analysis of intra-specific variation in the fatty acid profiles of Borrelia burgdorferi. J. Gen. Microbiol. 139:2197-2201.

Luft, B. J., P. D. Gorevic, J. J. Halperin, D. J. Volkman, and R. J. Dattwyler. 1989. A perspective on the treatment of Lyme borreliosis. Rev. Infect. Dis. 11(suppl. 6):S1518-1525.

Ma, B., B. Christen, D. Leung, and C. Vigo-Pelfrey. 1992. Serodiagnosis of Lyme borreliosis by western immunoblot: reactivity of various significant antibodies against Borrelia burgdorferi. J. Clin. Microbiol. 30(2):370-376.

Ma, Y., A. Sturrock, and J. J. Weiss. 1991. Intracellular localization of Borrelia burgdorferi within human endothelial cells. Infect. Immun. 59(2):671-678.

MacDonald, A. B. 1986. Human fetal borreliosis, toxemia of pregnancy, and fetal death. Zbl. Bakt. Hyg. A 263:189-200.

MacDonald, A. B. 1989. Gestational Lyme borreliosis: implications for the fetus. Rheum. Dis. Clin. North Am. 15:657-677.

MacDonald, A. B., B. W. Berger, and T. G. Schwan. 1990. Clinical implications of delayed growth of the Lyme borreliosis spirochete, Borrelia burgdorferi. Acta Trop. (Basel) 48:89-94.

Magnarelli, L. A. 1988. Serologic diagnosis of Lyme disease. Ann. N. Y. Acad. Sci. 539:154-161.

Magnarelli, L. A. 1995. Current status of laboratory diagnosis for Lyme disease. Am. J. Med. 98(4A)10S-12S.

Magnarelli, L. A. and J. F. Anderson. 1988a. Enzyme-linked immunosorbent assays for the detection of class-specific immunoglobulins to Borrelia burgdorferi. Am. J. Epidemiol. 127(4):818-825.

Magnarelli, L. A. and J. F. Anderson. 1988b. Ticks and biting insects infected with the etiologic agent of Lyme disease, Borrelia burgdorferi. J. Clin. Microbiol. 26(8):1482-1486.

Magnarelli, L. A., J. F. Anderson, and A. G. Barbour. 1986. The etiologic agent of Lyme disease in deer flies, horse flies, and mosquitoes. J. Infect. Dis. 154:355-358.

Magnarelli, L. A., J. F. Anderson, and A. G. Barbour. 1989. Enzyme-linked immunosorbent assays for Lyme disease: reactivity of subunits of Borrelia burgdorferi. J. Infect. Dis. 159(1):43-49.

Magnarelli, L. A., J. F. Anderson, and R. C. Johnson. 1987a. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. J. Infect. Dis. 156(1):183-189.

Magnarelli, L. A., J. F. Anderson, and R. C. Johnson, R. B. Nadelman, and G. P. Wormser. 1994. Comparison of different strains of Borrelia burgdorferi sensu lato used as antigens in enzyme-linked immunosorbent assays. J. Clin. Microbiol. 32(5):1154-1158.

Magnarelli, L. A., J. F. Anderson, J. F. Kaufmann, L. L. Lieberman, and G. D. Whitney. 1988c. Borreliosis in dogs from southern Connecticut. J. Am. Vet. Med. Assoc. 186:955-959.

Magnarelli, L. A., J. F. Anderson, A. B. Schreier, and C. M. Ficke. 1987b. Clinical and serologic studies of canine borreliosis. J. Am. Vet. Med. Assoc. 191(9):1089-1094.

Magnarelli, L. A., J. E. Freier, and J. F. Anderson. 1987c. Experimental infections of mosquitoes with Borrelia burgdorferi the etiologic agent of Lyme disease. J. Infect. Dis. 156(4):694-695.

Magnarelli, L. A. and K. C. Stafford III. 1992. Borrelia burgdorferi in Ixodes dammini (Acari: Ixodidae) feeding on birds in Lyme, Connecticut, U. S. A. Can. J. Zool. 70:2322-2325.

Malloy, D. C., R. K. Nauman, and H. Paxton. 1990. Detection of Borrelia burgdorferi using the polymerase chain reaction. J. Clin. Microbiol. 28:1089-1093.

Marconi, R. T. and C. F. Garon. 1992. Phylogenetic analysis of the genus Borrelia: a comparison of North American and European isolates of Borrelia burgdorferi. J. Bacteriol. 174:241-244.

Marconi, R. T., L. Lubke, W. Hauglum, and C. F. Garon. 1992. Species-specific identification of and distinction between Borrelia burgdorferi genomic groups by using 16S rRNA-directed oligonucleotide probes. J. Clin. Microbiol. 30(3):628-632.

Marshall III, W. F., S. R. Telford III, P. N. Rys, B. J. Rutledge, D. Mathiesen, S. E. Malawista, A. Spielman, and D. H. Persing. 1994. Detection of Borrelia burgdorferi DNA in museum specimens of Peromyscus leucopus. J. Infect. Dis. 170:1027-1032.

Masuzawa, T., H. Suzuki, H. Kawabata, F. Ishiguro, N. Takada, Y. Yano, and Y. Yanagihara. 1995. Identification of spirochetes isolated from wild rodents in Japan as Borrelia japonica. J. Clin. Microbiol. 33(5):1392-1394.

Mather, T. N., D. C. Duffy, and S. R. Campbell. 1993. An unexpected result from burning vegetation to reduce Lyme disease transmission risks. J. Med. Entomol. 30(3):642-645.

Mather, T. N., J. Piesman, and A. Spielman. 1987a. Absence of spirochetes (Borrelia burgdorferi) and piroplasms (Babesia microti) in deer ticks (Ixodes dammini) parasitized by chalcid wasps (Hunterellus hookeri). Med Vet. Entomol. 1:3-8.

Mather, T. N., J. M. C. Ribeiro, S. I. Moore, and A. Spielman. 1988. Reducing transmission of Lyme disease spirochetes in a suburban setting. Ann. N. Y. Acad. Sci. 539:402-403.

Mather, T. N., J. M. C, Ribeiro, and A. Spielman. 1987b. Lyme disease and babesiosis: acaricide focused on potentially infected ticks. Am J. Trop. Med Hyg. 33:609-614.

Mather, T. N., S. R. Telford III, and G. H. Adler. 1991. Absence of transplacental transmission of Lyme disease spirochetes from reservoir mice (Peromyscus leucopus) to their offspring. J. Infect. Dis. 164(3):564-567.

Mather, T. N., M. L. Wilson, S. I. Moore, J. M. C. Ribeiro, and A. Spielman. 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (Borrelia burgdorferi). Am. J. Epidemiol. 130:143-150.

Matuschka, F. R. and A. Spielman. 1986. The emergence of Lyme disease in a changing environment in North America and central Europe. Exp. Appl. Acarol. 2:337-353.

Maupin, G. O., D. Fish, J. Zultowsky, E. Campos, and J. Piesman. 1991. Landscape ecology of Lyme disease in a residential area of Westchester County, New York. Am. J. Epidemiol. 133(11):1105-1113.

McLean, R. G., S. R. Ubico, and L. M. Cooksey. 1993a. Experimental infection of the eastern chipmunk (Tamias striatus) with the Lyme disease spirochete (Borrelia burgdorferi). J. Wildl. Dis. 29(4):527-532.

McLean, R. G., S. R. Ubico, C. A . Norton Hughes, S. M. Engstrom, and R. C. Johnson. 1993b. Isolation and characterization of Borrelia burgdorferi from blood of a bird captured in Saint Croix River Valley. J. Clin. Microbiol. 31(8):2038-2043.

Mitchell, P. D., K. D. Reed, T. L. Aspeslet, M. F. Vandermause, and J. W. Melski. 1994. Comparison of four immunoserologic assays for detection of antibodies to Borrelia burgdorferi in patients with culture-positive erythema migrans. J. Clin. Microbiol. 32(8):1958-1962.

Mitchell, P. D., K. D. Reed, M. F. Vandermause, and J. W. Melski. 1993. Isolation of Borrelia burgdorferi from skin biopsy specimens of patients with erythema migrans. Am. J. Clin. Pathol. 99(1):104-107.

Moody, K. D., S. W. Barthold, and G. A. Terwilliger. 1990. Lyme borreliosis in laboratory animals: effect of host species and in vitro passage of Borrelia burgdorferi. Am. J. Trop. Med. Hyg. 43(1):87-92.

Morshed, M. G., H. Konishi, T. Nishimura, and T. Nakazawa. 1993. Evaluation of agents for use in medium for selective isolation of Lyme disease and relapsing fever Borrelia species. Eur. J. Clin. Microbiol. Infect. Dis. 12(7):512-518.

Moter, S. E., H. Hofmann, R. Wallich, M. M. Simon, and M. D. Kramer. 1994. Detection of Borrelia burgdorferi sensu lato in lesional skin of patients with erythema migrans and acrodermatitis chronica atrophicans by opsA-specific PCR. J. Clin. Microbiol. 32(12):2980-2988.

Nadelman, R. B., J. Nowakowski, G. Forseter, S. Bittker, D. Cooper, N. Goldberg, D. McKenna, and G. P. Wormser. 1993. Failure to isolate Borrelia burgdorferi after antimicrobial therapy in culture-documented Lyme borreliosis associated with erythema migrans: report of a prospective study. Am. J. Med. 94(6):583-588.

Nadelman, R. B., C. S. Pavia, L. A. Magnarelli, and G. P. Wormser. 1990. Isolation of Borrelia burgdorferi from the blood of seven patients with Lyme disease. Am. J. Med. 88:21-26.

Nadelman, R. B. and G. P. Wormser. 1995. Erythema migrans and early Lyme disease. Am. J. Med. 98(4A):15S-23S.

Nakao, M., K. Miyamoto, and M. Fukunaga. 1994. Lyme disease spirochetes in Japan: enzootic transmission cycles in birds, rodents, and Ixodes persulcatus ticks. J. Infect. Dis. 170:878-882.

National Committee for Clinical Laboratory Standards. 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically - second edition; Approved Standard. NCCLS Document M7-A2. Villanova, Pa.

Needham, Glen R. 1984. Evaluation of five popular methods for tick removal. Pediatrics 75(6):997-1002.

Nelson, J. A., J. K. Bouseman, U. Kitron, S. M. Callister, B. Harrison, M. J. Bankowski, M. E. Peeples, B. J. Newton, and J. F. Anderson. 1991. Isolation and characterization of Borrelia burgdorferi from Illinois Ixodes dammini. J. Clin. Microbiol. 29(8):a-c.

Neubert, U., M. Schaller, E. Januschke, W. Stolz, and H. Schmieger. 1993. Bacteriophages induced by ciprofloxacin in a Borrelia burgdorferi skin isolate. Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. 279(3):307-315.

Nguyen, T.-P. K., T. T. Lam, S. W. Barthold, S. R. Telford III, R. A. Flavell, and E. Fikrig. 1994. Partial destruction of Borrelia burgdorferi within ticks that engorged on OspE- or OspF-immunized mice. Infect. Immun. 62(5):2079-2084.

Nocton, J. J., F. Dressler, B. J. Rutledge, P. N. Rys, D. H. Persing, and A. C. Steere. 1994. Detection of Borrelia burgdorferi DNA by polymerase chain reaction in synovial fluids from patients with Lyme arthritis. N. Engl. J. Med. 330:229-234.

Noguchi, H. 1912. The pure cultivation of Spirochaeta duttoni, Spirocheata kochi, Spirocheata obermeieri, and Spirocheata novy. J. Exp. Med. 16:199-212.

Norris, S. J., C. J. Carter, J. K. Howell, and A. G. Barbour. 1992. Low-passage-associated proteins of Borrelia burgdorferi B31: characterization and molecular cloning of ospD, a surface-exposed, plasmid-encoded lipoprotein. Infect. Immun. 60(11):4662-4672.

Norris, C., A. M. Pappenheimer, and T. Flournoy. 1906. Study of a spirochete obtained from a case of relapsing fever in man, with notes on morphology, animal reactions, and attempts at cultivation. J. Infect. Dis. 3:266-290.

Novy, F.G. and R.E. Knapp. 1906. The cultivation of Spirillium obermeier. JAMA. 26: 2152-2154.

Oag, R. K. 1939. The growth of Borrelia duttoni in the developing egg. J. Pathol. Bacteriol. 49:339-344.

Oliver Jr., J. H., F. W. Chandler Jr., A. M. James, F. H. Sanders Jr., H. J. Hutchenson, L. O. Huey, B. S. McGuire, and R. S. Lane. 1995. Natural occurence and characterization of the Lyme disease spirochete, Borrelia burgdorferi, in cotton rats (Sigmodon hispidus) from Georgia and Florida. J. Parasitol. 81(1):30-36.

Oliver Jr., J. H., M. R. Owsley, H. J. Hutcheson, A. M. James, C. Chen, W. S. Irby, E. M. Dotson, and D. K. McLain. 1993. Conspecificity of the ticks Ixodes scapularis and I. dammini (Acari: Ixodidae). J. Med. Entomol. 30:54-62.

Osebold, J. W., R. Spezialetti, M. B. Jennings, R. F. Pritchett, and R. B. Bushnell. 1986. Congenital spirochetosis in calves: association with epizootic bovine abortion. J. Am. Vet. Med. Assoc. 188:371-376.

Pachner, A. R., J. Basta, E. Delaney, and D. Hulinska. 1995. Localization of Borrelia burgdorferi in murine Lyme borreliosis by electron microscopy. Am. J. Trop. Med. Hyg. 52(2):128-133.

Pachner, A. R. and E. Delaney. 1993. Murine Lyme borreliosis: route of inoculation determines immune response and infectivity. Reg. Immunol. 4:345-351.

Pachner, A. R., N. Ricalton, and E. Delaney. 1993. Comparison of polymerase chain reaction with culture and serology for diagnosis of murine experimental Lyme borreliosis. J. Clin. Microbiol. 31(2):208-214.

Padula, S. J., F. Dias, A. Sampieri, R. B. Craven, and R. W. Ryan. 1994. Use of recombinant OspC from Borrelia burgdorferi for serodiagnosis of early Lyme disease. J. Clin. Microbiol. 32(7):1733-1738.

Padula, S. J., A. Sampieri, F. Dias, A. Szczepanski, and R. W. Ryan. 1993. Molecular characterization and expression of p23 (OspC) from a North American strain of Borrelia burgdorferi. Infect. Immun. 61:5097-5105.

Parker, J. L. and K. H. White. 1992. Lyme borreliosis in cattle and horses: a review of the literature. Cornell Vet. 82:253-274.

Pavia, C. S. and S. Bittker. 1988. Assessment of in vitro growth of Borrelia burgdorferi by tritiated adenine incorporation. Ann. N. Y. Acad. Sci. 539:410-413.

Persing, D. H., S. R. Telford III, P. N. Rys, D. E. Dodge, T. J. White, S. E. Malawista, A. Spielman. 1990a. Detection of Borrelia burgdorferi DNA in museum specimens of Ixodes dammini ticks. Science 249:1420-1423.

Persing, D. H., S. R. Telford III, A. Spielman, and S. W. Barthold. 1990b. Detection of Borrelia burgdorferi infection in Ixodes dammini ticks with the polymerase chain reaction. J. Clin. Microbiol. 28(3):566-572.

Pfister, H. W., U. Neubert, B. Wilske, V. Preac-Mursic, K. M. Einhaupl, and G. D. Borasio. 1986. Reinfection with Borrelia burgdorferi. Lancet 2:984-985

Pflueger, K. H., C. D. Reimers, U. Neubert, C. Meisel, B. Trapp, J. Leititis, B. Voelker, P. Muenchoff, and J. Litzenberger. 1989. Lyme-borreliosis and possible association with HLA-antigens. Tissue Antigens 33(3):375-381.

Philipson, A. 1991. Antibiotic treatment in Lyme borreliosis. Scand. J. Infect. Dis. Suppl. 77:145-150.

Picken, R. 1992. Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. J. Clin. Microbiol. 33:166-172.

Piesman, J. 1989. Transmission of Lyme disease spirochetes (Borrelia burgdorferi). Exp. Appl. Acarol. 7(1):71-80.

Piesman, J. 1991. Experimental acquisition of the Lyme disease spirochete, Borrelia burgdorferi, by larval Ixodes dammini (Acari: Ixodidae) during partial blood meals. J. Med. Entomol. 28(2):259-262.

Piesman, J. 1993. Standard system for infecting ticks (Acari: Ixodidae) with the Lyme disease spirochete, Borrelia burgdorferi. J. Med. Entomol. 30(1):199-203.

Piesman, J. 1995. Dispersal of the Lyme disease spirochete Borrelia burgdorferi to salivary glands of feeding nymphal Ixodes scapularis (Acari: Ixodidae). J. Med. Entomol. 32(4):519-521.

Piesman, J., T. N. Mather, G. J. Dammin, S. R. Telford III, C. C. Lastavica, and A. Spielman. 1987a. Seasonal variation of transmission risk of Lyme disease and human babesiosis. Ann. J. Epidem. 126(6):1187-1189.

Piesman, J., T. N. Mather, R. J. Sinsky, and A. Spielman. 1987b. Duration of tick attachment and Borrelia burgdorferi transmission. J. Clin. Microbiol. 25:557-558.

Piesman, J., G. O. Maupin, E. G. Campos, and C. M. Happ. 1991. Duration of adult female Ixodes dammini attachment and transmission of Borrelia burgdorferi, with description of a needle aspiration isolation method. J. Infect. Dis. 163(4):895-897.

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(4):352-357.

Platt, K. B., M. G. Novak, and W. A. Rowley. 1992. Studies on the biology of Ixodes dammini in the upper midwest of the United States. Ann. N. Y. Acad. Sci. 653:78-87.

Pollack, R. J., S. R. Telford III, and A. Spielman. 1993. Standardization of medium for culturing Lyme disease spirochetes. J. Clin. Microbiol. 31(5):1251-1255.

Preac-Mursic, V., K. Weber, H. W. Pfister, B. Wilske, B. Gross, A. Baumann, and J. Prokop. 1989. Survival of Borrelia burgdorferi in antibiotically treated patients with Lyme borreliosis. Infection. 17:355-359.

Preac-Mursic, V., B. Wilske, and S. Reinhardt. 1991. Culture of Borrelia burgdorferi on six solid media. Eur. J. Clin. Microbiol. Infect. Dis. 10:1076-1079.

Preac-Mursic, V., B. Wilske, and G. Schierz. 1986. European Borrelia burgdorferi isolated from humans and ticks, culture conditions, and antibiotic susceptibility. Zbl. Bakt. Hyg. A 263:112-118.

Preac-Mursic, V., B. Wilske, G. Schierz, M. Holmburger, and E. Suess. 1987. In vitro and in vivo susceptibility of Borrelia burgdorferi. Eur. J. Clin. Microbiol. 6(4):424-426.

Radda, A., I. Burger, G. Stanek, and G. Wewalka. 1986. Austrian hard ticks as vectors of Borrelia burgdorferi. Zbl. Bakt. Hyg. A 263:79-82.

Radolf, J. D., K. W. Bourell, D. R. Akins, J. S. Brusca, and M. V. Norgard. 1994. Analysis of Borrelia burgdorferi membrane architecture by freeze-fracture electron microscopy. J. Bacteriol. 176(1):21-31.

Rahn, D. W. 1991. Lyme disease: clinical manifestations, diagnosis, and treatment. Semin. Arth. Rheum. 20(4):201-218.

Rahn, D. W. 1992. Treating Lyme disease. Postgrad. Med. 91(7):57-64.

Rahn, D. W. and S. E. Malawista. 1991. Lyme disease: recommendations for the diagnosis and treatment. Ann. Intern. Med. 114:472-481.

Rand, P. W., R. P. Smith Jr., and E. H. Lacombe. 1991. Canine seroprevalence and the distribution of Ixodes dammini in an area of emerging Lyme disease. Am. J. Pub. Health. 81(10):1331-1334.

Randolph, S. E. and P. A. Nuthall. 1994. Nearly right or precisely wrong? Natural versus laboratory studies of vector-borne diseases. Parasit. Today 10(2):458-462.

Rawlings, J. A., P. V. Fournier, and G. J. Teltow. 1987. Isolation of Borrelia spirochetes from patients in Texas. J. Clin. Microbiol. 25(7):1148-1150.

Rawlings, J. A. and G. J. Teltow. 1994. Prevalence of Borrelia (Spirochaetaceae) spirochetes in Texas ticks. J. Med. Entomol. 31(2):297-301.

Reik, L. 1991. Lyme Disease and the Nervous System. Theime Medical Publishers, Inc. New York.

Reiss-Gutfreund, R. J. 1960. Culture de Borrelia recurrentis (souches ethiopiénnes) en oeuf féconde de poule. Ann. Inst. Pasteur 98:131-136.

Reznick, J. W., D. B. Braunstein, and R. L. Walsh. 1986. Lyme carditis: electrophysiology and histopathologic study. Am. J. Med. 81:923-927.

Ribeiro, J. M. C., T. N. Mather, J. Piesman, and A. Spielman. 1987. Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari:Ixodidae). J. Med. Entomol. 24:201-205.

Roehrig, J. T., J. Piesman, A. R. Hunt, M. G. Keen, C. M. Happ, and B. J. B. Johnson. 1992. The hamster immune response to tick-transmitted Borrelia burgdorferi differs from the response to needle-inoculated cultured organisms. J. Immun. 149(11):3648-3653.

Roessner, K., E. Fikrig, J. Q. Russell, S. M. Cooper, R. A. Flavell, and R. C. Budd. 1994. Prominent T lymphocyte response to Borrelia burgdorferi from peripheral blood of unexposed donors. Eur. J. Immunol. 24(2):320-324.

Rosa, P. A., D. Hogan, and T. G. Schwan. 1991. Polymerase chain reaction analyses identify two distinct classes of Borrelia burgdorferi. J. Clin. Microbiol. 29:524-532.

Rosa, P. A. and T. G. Schwan. 1989. A specific and sensitive assay for the Lyme disease spirochete Borrelia burgdorferi using the polymerase chain reaction. J. Infect. Dis. 160(6):1018-1029.

Rosa, P. A., T. G. Schwan, and D. Hogan. 1992. Recombination between genes encoding major outer surface proteins A and B of Borrelia burgdorferi. Mol. Microbiol. 6(20):3031-3040.

Ruberti, G., A. B. Begovich, A. C. Steere, W. Klitz, H. A. Erlich, and C. G. Fathman. 1991. Molecular analysis of the role of the HLA class II genes DRB1, DQA1, DQB1, and DPB1 in susceptibility to Lyme arthritis. Hum. Immunol. 31(1):20-27.

Russell, H., J. S. Sampson, G. P. Schmid, H. W. Wilkinson, and B. Plikaytis. 1984. Enzyme linked immunosorbent assay and indirect immunofluorescence assay for Lyme disease. J. Infect. Dis. 149:465-470.

Sadziene, A., P. A. Rosa, P. A. Thompson, D. M. Hogan, and A. G. Barbour. 1992. Antibody resistant mutants of Borrelia burgdorferi: in vitro selection and characterization. J. Exp. Med. 176:799-809.

Sambri, V., C. Stefanelli, and R. Cevenini. 1992. Detection of glycoproteins in Borrelia burgdorferi. Arch. Microbiol. 157(3):205-208.

Schlesinger, P. A., P. H. Duray, B. A. Burke, A. C. Steere, and M. T. Stillman. 1985. Maternal-fetal transmission of the Lyme disease spirochete, Borrelia burgdorferi. Ann Intern. Med. 103:67-68.

Schmid, G. P. 1985. The global distribution of Lyme disease. Rev. Infect. Dis. 7(1):41-50.

Schmid, G. P. 1989. Epidemiology and clinical similarities of human spirochetal diseases. Rev. Infect. Dis. 11 (suppl. 6): S1460-S1469.

Schmid, G. P., A. G. Steigerwalt, S. E. Johnson, A. G. Barbour, A. C. Steere, I. M. Robinson, and D. J. Brenner. 1984. DNA characteristics of Lyme disease spirochetes. Yale J. Biol. Med. 57:539-542.

Schmidli, J., T. Hunziker, P. Moesli, and U. B. Schaad. 1988. Cultivation of Borrelia burgdorferi from the joint fluid three months after treatment of facial palsy due to Lyme borreliosis. J. Infect. Dis. 158(4):905-906.

Schmitz, J. L., C. S. Powell, and J. D. Folds. 1993. Comparison of seven commercial kits for detection of antibodies to Borrelia burgdorferi. Eur. J. Clin. Microbiol. Infect. Dis. 12(6):419-424.

Schned, E. S. and D. N. Williams. 1992. Concerns in Lyme disease. Postgrad. Med. 91(7):65-70.

Schoeler, G. B. and R. S. Lane. 1993. Efficiency of transovarial transmission of the Lyme disease spirochete, Borrelia burgdorferi, in the western black-legged tick, Ixodes pacificus. J. Med. Entomol. 30(1):80-86.

Schreck, C. E., E. L. Snoddy, and A. Spielman. 1986. Pressurized sprays of permethrin or deet on military clothing for personal protection against Ixodes dammini (Acari:Ixodidae). J. Med. Entomol. 23:396-399.

Schubach, W. H., S. Mudri, R. J. Dattwyler, and B. J. Luft. 1991. Mapping antibody-binding domains of the major outer surface membrane protein (OspA) of Borrelia burgdorferi. Infect. Immun. 59(6):1911-1915.

Schulze, T. L., W. M. McDevitt, W. E. Parkin, and J. K. Shisler. 1987. Effectiveness of two insecticides in controlling Ixodes dammini (Acari: Ixodidae) following an outbreak of Lyme disease in New Jersey. J. Med. Entomol. 24:420-424.

Schutzer, S. E., P. K. Coyle, A. L. Belman, M. G. Golightly, and J. Drulle. 1990. Sequestration of antibody to Borrelia burgdorferi in immune complexes in seronegative Lyme disease. Lancet 1:312-315.

Schutzer, S. E., P. K. Coyle, J. J. Dunn, B. J. Luft, and M. Brunner. 1994. Early and specific antibody response to OspA in Lyme disease. J. Clin. Invest. 94:454-457.

Schwan, T. G. and W. Burgdorfer. 1987. Antigenic changes of Borrelia burgdorferi as a result of in vitro cultivation. J. Infect. Dis. 156(5):852-853.

Schwan, T. G., W. Burgdorfer, and C. F. Garon. 1988a. Changes in infectivity and plasmid profile of the Lyme disease spirochete, Borrelia burgdorferi, as a result of in vitro cultivation. Infect. Immun. 56(8):1831-1836.

Schwan, T. G., W. Burgdorfer, M. E. Schrumpf, and R. H. Kartens. 1988b. The urinary bladder, a consistent source of Borrelia burgdorferi in experimentally infected white-footed mice (Peromyscus leucopus). J. Clin. Microbiol. 26(5):893-895.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa. 1995. Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. Proc. Natl. Acad. Sci. USA 92(7):2909-2913.

Schwan, T. G., M. E. Schrumpf, R. H. Karstens, J. R. Clover, J. Wong, M. Daugherty, M. Struthers, and P. R. Rosa. 1993. Distribution and molecular analysis of Lyme disease spirochetes, Borrelia burgdorferi, isolated from ticks throughout California. J. Clin. Microbiol. 31(12):3096-3108.

Schwan, T. G. and W. J. Simpson. 1991. Factors influencing the antigenic reactivity of Borrelia burgdorferi, the Lyme disease spirochete. Scand. J. Infect. Dis. Suppl. 77:94-101.

Schwan, T. G., W. J. Simpson, M. E. Schrumpf, and R. H. Kartens. 1989. Identification of Borrelia burgdorferi and B. hermsii using DNA hybridization probes. J. Clin. Microbiol. 27:1734-1738.

Schwartz, I. S. Bittker, S. L. Bowen, D. Cooper, C. Pavia, and G. P. Wormser. 1993. Polymerase chain reaction amplification of culture supernatants for rapid detection of Borrelia burgdorferi. Eur. J. Clin. Microbiol. Infect. Dis. 12(11):879-882.

Shadick, N. A., C. B. Phillips, E. L. Logigian, A. C. Steere, R. F. Kaplan, V. P. Berardi, P. H. Duray, M. G. Larson, E. A. Wright, K. S. Ginsburg, J. N. Katz, and M. H. Liang. 1994. The long term clinical outcomes of Lyme disease. Ann. Intern. Med. 121:56-567.

Shapiro, E. D., M. A. Gerber, N. B. Holabird, A. T. Berg, H. M. Feder, G. L. Bell, P. N. Rys, and D. H. Persing. 1992. A controlled trial of antimicrobial prophylaxis for Lyme disease after deer-tick bites. N. Engl. J. Med. 327(25):1769-1773.

Shiffman, M. L., F. B. Keith, and E. W. Moore. 1990. Pathogenesis of ceftriaxone-associated biliary sludge. Gastroenterology 99:1772-8.

Shih, C. M., R. J. Pollack, S. R. Telford III, and A. Spielman. 1992. Delayed dissemination of Lyme disease spirochetes from the site of deposition in the skin of mice. J. Infect. Dis. 166: 827-831.

Shih, C. M. and A. Spielman. 1993. Topical prophylaxis for Lyme disease after tick bite in a rodent model. J. Infect. Dis. 168:1042-1045.

Shih, C. M., A. Spielman, and S. R. Telford III. 1995a. Short report: mode of action of protective immunity to Lyme disease spirochetes. Am. J. Trop. Med. Hyg. 52(1):72-74.

Shih, C. M., S. R. Telford III, and A. Spielman. 1995b. Effect of ambient temperature on competance of deer ticks as hosts for Lyme disease spirochetes. J. Clin. Microbiol. 33(4):958-961.

Shoberg, R. J. and D. D. Thomas. 1993. Specific adherence of Borrelia burgdorferi extracellular vesicles to human endothelial cells in culture. Infect. Immun. 61(9):3892-3900.

Shrestha, M., R. L. Grodzicki, and A. C. Steere. 1985. Diagnosing early Lyme disease. Am J. Med. 78:235-240.

Sigal, L. H. 1990. Summary of the first 100 patients seen at a Lyme disease referrral center. Am. J. Med. 88:577-581.

Sigal, L. H., A. C. Steere, D. H. Freeman, and J. M. Dwyer. 1986. Proliferative responses of mononuclear cells in Lyme disease: reactivity to Borrelia burgdorferi antigens is greater in joint fluid than in blood. Arthritis Rheum. 29(6):761-769.

Sigal, L. H. and A. Tatum. 1988. Lyme disease patients' sera contains IgM antibodies to Borrelia burgdorferi that cross-react with neuronal antigens. Neurology 38:1439-1442.

Simon, M. M., U. E. Schaible, M. D. Kramer, C. Eckerskorn, C. Museteanu, H. K. Muller-Hermelink, and R. Wallich. 1991. Recombinant outer surface protein A from Borrelia burgdorferi induces antibodies protective against spirochetal infection in mice. J. Infect. Dis. 164(1):123-132.

Simpson, W. J., W. Burgdorfer, M. E. Schrumpf, R. H. Karstens, and T. G. Schwan. 1991a. Antibody to a 39-kilodalton Borrelia burgdorferi antigen (P39) as a marker for infection in experimentally and naturally inoculated animals. J. Clin. Microbiol. 29(2):236-243.

Simpson, W. J., C. F. Garon, and T. G. Schwan. 1990. Analysis of supercoiled circular plasmids in infectious and non-infectious Borrelia burgdorferi. Microb. Pathog. 8(2):109-118.

Simpson, W. J., M. E. Schrumpf, S. F. Hayes, and T. G. Schwan. 1991b. Molecular and immunological analysis of a polymorphic periplasmic protein of Borrelia burgdorferi. J. Clin. Microbiol. 29(9):1940-1948.

Sinsky, R. J. and J. Piesman. 1989. Ear punch biopsy method for detection and isolation of Borrelia burgdorferi from rodents. J. Clin. Microbiol. 27(8):172-177.

Smith, R. P., P. W. Rand, E. H. Lacombe, S. R. Telford III, S. M. Rich, J. Piesman, and A. Spielman. 1993. Norway rats as reservoirs for Lyme disease spirochetes on Monhegan Island, Maine. J. Infect. Dis. 168:687-691.

Snydman, D. R., D. P. Schenkein, V. P. Berardi, C. C. Lastavica, and K. M. Pariser. 1986. Borrelia burgdorferi in joint fluid in chronic Lyme arthritis. Ann. Intern. Med. 104:798-800.

Sonenshine, D. E. and G. Haines. 1985. A convenient method for controlling populations of the American dog tick, Dermacentor variabilis (Acari:Ixodidae), in the natural environment. J. Med. Entomol. 22:577-583.

Spielman, A. 1988. Prospects for suppressing transmission of Lyme disease. Ann. N. Y. Acad. Sci. 539:212-220.

Spielman, A., J. F. Levine, and M. L. Wilson. 1984. Vectorial capacity of North American Ixodes ticks. Yale J. Biol. Med. 57:507-513.

Stafford, K. C. 1989. Lyme disease prevention: personal protection and prospects for tick control. Conn. Med. 53:347-351.

Stalhammar-Carlemalm, M., E. Jenny, L. Gern, A. Aeschlimann, and J. Meyer. 1990. Plasmid analysis and restriction fragment length polymorphisms of chromosomal DNA allow a distinction between Borrelia burgdorferi strains. Zentralbl. Bakteriol. 274:28-39.

Stanek, G., I. Burger, A. Hirschl, G. Wewalka, and A. Radda. 1986. Borrelia transfer by ticks during their life cycle. Zbl. Bakt. Hyg. A 263:29-33.

Stanek, G., Klein, J., R. Bittner, and D. Glogar. 1990. Isolation of Borrelia burgdorferi from the myocardium of a patient with longstanding cardiomyopathy. N. Engl. J. Med. 322(1):249-252.

Stanek, G., G. Wewalka, V. Groh, R. Neumann, and W. Kristoferitsch. 1985. Differences between Lyme disease and European arthropod-borne borrelia infections. Lancet i:401.

Steere, A. C. 1989. Lyme disease. N. Engl. J. Med. 321(9):586-596.

Steere, A. C. 1991. Clinical definitions and differential diagnosis of Lyme arthritis. Scand. J. Infect. Dis. suppl. 51-51.

Steere, A. C., E. Dwyer, and R. Winchester. 1990. Association of chronic Lyme arthritis with HLA-DR4 and HLA-DR2 alleles. N. Engl. J. Med. 323(4):219-223.

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(13):732-742.

Steere, A. C., and S. E. Malawista. 1979. Cases of Lyme disease in the United States: locations correlated with distribution of Ixodes dammini. Ann. Intern. Med. 91:730-733.

Stiernstedt, G., R. Dattwyler, P. H. Durray, K Hansen, J. Jirous,R. C. Johnson, M. Karlsson, V. Preac-Mursic, and T. G. Schwan. 1991. Diagnostic tests in Lyme borreliosis. Scand. J. Infect. Dis. Suppl. 77:136-142.

Stobierski, M. G., W. N. Hall, B. Robinson-Dunn, H. Stiefel, S. Shiflett, and V. Carlson. 1994. Isolation of Borrelia burgdorferi from two patients in Michigan. Clin. Infect. Dis. 19(5):891-896.

Stoenner, H. G. 1974. Biology of Borrelia hermsii in Kelly medium. Appl. Microbiol. 28(4):540-543.

Stoenner, H. G., T. Dodd, and C. Larsen. 1982. Antigenic variation of Borrelia hermsii. J. Exp. Med. 156:1297-1311.

Szczepanski, A., M. B. Furie, J. L. Benach, B. P. Lane, and H. B. Fleit. 1990. Interaction between Borrelia burgdorferi and endothelium in vitro. Am. Soc. Clin. Invest. 85:1637-1647.

Talice, R.C. and N. Surraco. 1929. Sur la culture du treponema hispanicum. Parasit. Humaine et Comparee. 7:133-139.

Tälleklint, L. and T. G. T. Jaenson. 1993. Maintenance by hares of European Borrelia burgdorferi in ecosystems without rodents. J. Med. Entomol. 30:439-460.

Telford III, S. R., E. Fikrig, S. W. Barthold, L. R. Brunet, A. Spielman, and R. A. Flavell. 1993. Protection against antigenically variable Borrelia burgdorferi conferred by recombinant vaccines. J. Exp. Med. 178:755-758.

Telford III, S. R., T. N. Mather, G. H. Adler, and A. Spielman. 1990. Short-tailed shrews as reservoirs of the agents of Lyme disease and human babesiosis. J. Parasitol. 76:681-683.

Thieking, A., S. M. Goyal, R. F. Bey, K. I. Loken, L. D. Mech, R. P. Thiel, and T. P. O'Connor. 1992. Seroprevalence of Lyme disease in gray wolves from Minnesota and Wisconsin. J. Wildl. Dis. 28:177-182.

Thomas. D. D., D. Cadavid, and A. G. Barbour. 1994. Differential association of Borrelia species with cultured neural cells. J. Infect. Dis. 169(2):445-448.

Tsai, T. F., R. E. Bailey, and P. S. Moore. 1989. National surveillance of Lyme disease, 1987-1988. Conn. Med. 53:324-326.

Van Dam, A. P., H. Kuiper, K. Vos, A. Widjojokusumo, B. M. de Jongh, L. Spanjaard, A. C. P. Ramselaar, M. D. Kramer, and J. Dankert. 1993. Different genospecies of Borrelia burgdorferi are associated with distinct clinical manifestations of Lyme borreliosis. Clin. Infect. Dis. 17:708-717.

Van Der Linde, M. R. 1991. Lyme carditis: clinical characteristic of 105 cases. Scand. J. Infect. Dis. Suppl. 77:81-84.

Volk, W. A. and M. F. Wheeler. 1988. Basic Microbiology. Sixth edition. p. 85-86. Harper and Row, New York.

Walker, E. D., T. S. Smith, J. DeWitt, D. C. Beaudo, and R. G. McLean. 1994. Prevalence of Borrelia burgdorferi in host-seeking ticks (Acari:Ixodidae) from a Lyme disease endemic area in northern Michigan. J. Med. Entomol. 31(4):524-528.

Weber, K., H. -J. Bratzke, U. Neubert, B. Wilske, and P. H. Duray. 1988. Borrelia burgdorferi in a newborn despite oral penicillin for Lyme borreliosis during pregnancy. Pediatr. Infect. Dis. 7:286-289.

Weber, K., G. Schierz, B. Wilske, U. Neubert, H. E. Krampitz, A. G. Barbour, and W. Burgdorfer. 1986. Reinfection in erythema migrans disease. Infection 14:38-41.

Welsh, J., C. Pretzman, D. Postic, I. S. Girons, G. Baranton, and M. McClelland. 1992. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves Borrelia burgdorferi into three distinct phyletic groups. Inter. J. Sys. Bact. 42(3):370-7.

Williams, L. R. and F. E. Austin. 1992. Hemolytic activity of Borrelia burgdorferi. Infect. Immun. 60(8):324-3230.

Wilske, B. 1988. Serodiagnosis of Lyme-borreliosis. Z. Hautkr. 63(6):511-514.

Wilske, B., J. F. Anderson, G. Baranton, A. G. Barbour, K. Hovind-Hougen, R. C. Johnson, and V. Preac-Mursic. 1991. Taxonomy of Borrelia spp. Scand J. Infect. Dis. Suppl. 77:108-129

Wilske, B., A. G. Barbour, S. Bergström, N. Burman, B. I. Restrepo, P. A. Rosa, T. Schwan, E. Soutschek, and R. Wallich. 1992. Antigenic variation and strain heterogeneity in Borrelia spp. Res. Microbiol. 143:583-596.

Wilske, B., S. Jauris-Heipke, R. Lobentanzer, I. Pradel, V. Preac-Mursic, D. Rossler, E. Soutschek, and R. C. Johnson. 1995. Phenotypic analysis of outer surface protein C (OspC) of Borrelia burgdorferi sensu lato by monoclonal antibodies: relationship to genospecies and OspA serotype. J. Clin. Microbiol. 33(1):103-109.

Wilske, B., V. Preac-Mursic, U. B. Gobel, B. Graf, S. Jauris, E. Soutschek, E. Schwab, and G. Zumstein. 1993. An OspA serotyping system for Borrelia burgdorferi based on reactivity with monoclonal antibodies and OspA sequence analysis. J. Clin. Microbiol. 31:340-350.

Wilske, B., V. Preac-Mursic, G. Schierz, and K. V. Busch. 1986. Immunochemical and immunological analysis of European Borrelia burgdorferi strains. Zbl. Bakt. Hyg. A 263:92-102.

Wilske, B., V. Preac-Mursic, G. Schierz, R. Kuhbeck, A. G. Barbour, and M. Kramer. 1988. Antigenic variability of Borrelia burgdorferi. Ann N. Y. Acad. Sci. 539:126-143.

Wilson, M.L. 1986. Reduced abundance of adult Ixodes dammini (Acari: Ixodidae) following destruction of vegetation. J. Econ. Entomol. 79:693-696.

Wilson, M.L., J.F. Levine, and A. Speilman. 1984. Effect of deer reduction on abundance of the deer tick (Ixodus dammini). Yale J. Biol. Med. 57: 697-705.

Wilson, M. L., T. S. Litwin, and T. A. Gavin. 1988. Microgeographic distribution of deer and of Ixodes dammini: options for reducing the risk of Lyme disease. Ann. N. Y. Acad. Sci. 539:437-439.

Wilson, M. L., T. S. Litwin, T. A. Gavin, M. C. Capkanis, D. G. MacLean, and A. spielman. 1990. Host-dependent differences in feeding and reproduction of Ixodes dammini (Acari: Ixodidae). J. Med. Entomol. 27:945-954.

Wilson, M. L. and A. Spielman. 1985. Seasonal activity of immature Ixodes dammini (Acari: Ixodidae). J. Med. Entomol. 22:408-414.

Wise, D. J. and T. L. Weaver. 1991. Detection of the Lyme disease bacterium, Borrelia burgdorferi, by using the polymerase chain reaction and a nonradioisotopic gene probe. J. Clin. Microbiol. 29:1523-1526.

Wormser, G. P., G. Forseter, D. Cooper, J. Nowakowski, R. B. Nadelman, H. Horowitz, I. Schwartz, S. L. Bowen, G. L. Campbell, and N. S. Goldberg. 1992. Use of a novel technique of cutaneous lavage for diagnosis of Lyme disease associated with erythema migrans. JAMA 268:1311-1313.

Yanagihara, Y., H. Kawabata, and T. Masuzawa. 1994. Characterization of Borrelia japonica isolated from Ixodes ovatus in Japan. Proceedings of the VIth International Conference on Lyme Borreliosis, Bologna, Italy, June 19-22. Adv. Lyme Borrel. Res. 1994:27-30.

Zingg, B. C., R. N. Brown, R. S. Lane, and R. B. LeFebvre. 1993. Genetic diversity among Borrelia burgdorferi isolates from wood rats and kangaroo rats in California. J. Clin. Microbiol. 31(12):3109-3114.

Zoller, L., S. Burkard, and H. Schafer. 1991. Validity of Western immunoblot band patterns in the serodiagnosis of Lyme borreliosis. J. Clin. Microbiol. 29(1):236-243.

Zoschke, D. C. 1992. Testing for Lyme disease. Postgrad. Med. 91(7):46-55.

Zoschke, D. C., A. A. Skemp, and D. L. Defosse. 1991. Lymphoproliferative responses to Borrelia burgdorferi in Lyme disease. Ann. Intern. Med. 114:285-289.

Zung, J. L., S. Lewengrub, M. A. Rudzinska, A. Spielman, S. R. Telford, and J. Piesman. 1989. Fine structural evidence for the penetration of the Lyme disease spirochete Borrelia burgdorferi through the gut and salivary tissues of Ixodes dammini. Can. J. Zool. 67:1737-1748.

GENERAL REFERENCES

Significance of this disease has prompted researchers to convene worldwide conferences on Lyme disease and related disorders. The findings of the first international conference on Lyme borreliosis were published in 1984 in the Yale Journal of Biology and Medicine (vol. 57). Annual conferences have followed. one of the most recent was a NATO advanced research workshop on Lyme borreliosis held in 1993 in London. The proceedings from this workshop were edited by J. S. Axford and D. H. E. Rees and published in 1994 by Plenum Press, NY. Other general references are:

Anderson, J. F. and L. A. Magnarelli. 1992. Epizootiology of Lyme disease and methods of cultivating Borrelia burgdorferi. Ann. N.Y. Acad. Sci. 653:52-63.

Aspects of Lyme borreliosis. 1993. K. Weber, W. Burgdorfer, G. Schierz (ed.). Springer - Verlag, Berlin.

Barbour, A. G. and D. Fish. 1993. The biological and social phenomenen of Lyme disease. Science. 260:1610-1616.

Kazmierczak, J. J., and J. P. Davis. 1992. Lyme disease: ecology, epidemiology, clinical spectrum, and management. Advances in Pediatrics. 39:207-255.

Lane, R. S., J. Piesman, and W. Burgdorfer. 1991. Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe. Annu. Rev. Entomol. 36:587-609.

Lyme disease. 1993. P. K. Coyle (ed). Mosby-Year Book, Inc. St. Louis.

Needham, G. R. and P. D. Teel. 1991. Off-host physiological ecology of ixodid ticks. Annu. Rev. Entomol. 36:659-681.