Study of BSK II and BSK H Media for Culturing Lyme Disease Spirochetes

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Abstract

Borrelia burgdorferi, the causative agent of Lyme disease, can be grown in vitro in Barbour-Stoenner-Kelly (BSK II) and modified Barbour-Stoenner-Kelly (BSK H) growth media. Two growth media, BSK II and BSK H, were compared with respect to their ability to support the growth of three *Borrelia* isolates: *B. burgdorferi* sensu stricto CA4, *B. bissettii* sp. nov. CA389, and *B. corieaceae* CA435. The growth kinetics of high-passage (P10) and low-passage (P7) *B. bissettii* CA389 isolates were compared to determine if adaptation to cultural media facilitates faster growth of the isolates. In addition, effects of gelatin on the bacterial growth were studied. All isolates tested grew significantly faster and appeared healthier in BSK H media. The replication rate of *B. bissettii* correlated with the number of passages in the cultural media. The results of this study also suggested that gelatin inhibits growth of *B. burgdorferi* CA4 and *B. bissettii* CA389 in BSK H and BSK II media and has no significant effect on the growth rate of *B. corieaceae* CA435 in BSK H.

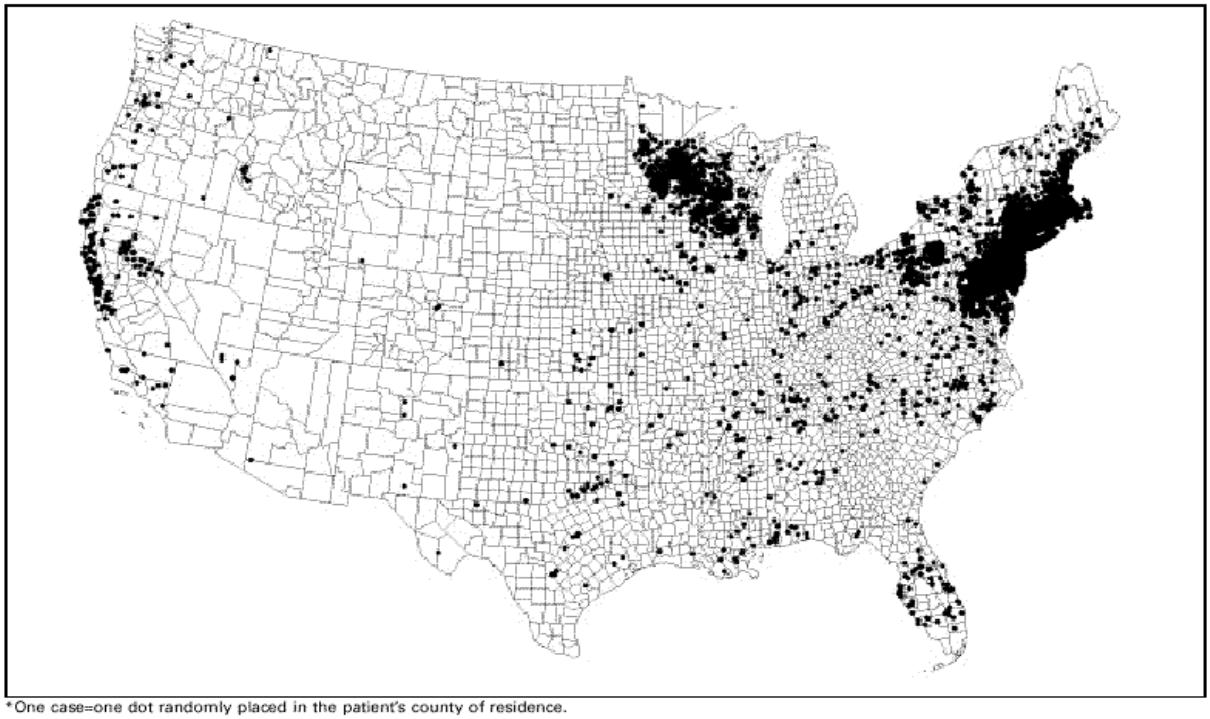
Introduction

With human population expansion and encroachment into wildlife habitats, people have entered a dangerous enzootic cycle causing them to become frequent accidental hosts for ticks and the disease agents they carry, including the bacterium *Borrelia burgdorferi*, the agent of Lyme disease (Daszak *et al.* 2000). Consequently, Lyme borreliosis has become the most frequently reported arthropod-born human infection in Europe and North America (Stanek *et al.* 1993). As of 1995, cases of Lyme disease had been reported in 48 of the 50 states and appear to be increasing, both in number of people affected and in geographic distribution, Figure 1 (Ostfeld 1997).

Lyme disease is transmitted by several tick species and caused by spirochetes from the general taxon *Borrelia burgdorferi* sensu lato (Postic *et al.* 1998). At least three genospecies including, B. *burgdorferi* sensu stricto, B. *garinii*, and B. *afzelii* are known to be associated with human disease in Europe (Baranton *et al.* 1992). In the United States B. *burgdorferi* sensu stricto has been the only genospecie known to cause Lyme borreliosis in the past; however, recent studies have shown substantial heterogeneity among Californian and other Northern American isolates (Mathiesen *et al.* 1997).

Figure 1. Lyme disease prevalence, United States, 1998 (CDC 1999).

LYME DISEASE — reported cases,* United States, 1998



*One case=one dot randomly placed in the patient's county of residence.

In 1998, a total of 16,801 cases of Lyme disease were reported, the highest number of cases ever reported. In December 1998, a new Lyme disease vaccine was approved by the U.S. Food and Drug Administration. The Advisory Committee on Immunization Practices (ACIP) issued recommendations for the use of this vaccine in June 1999 (*MMWR* 1999;48[RR-7]).

Using the nucleotide sequence analysis of ribosomal gene from 19 atypical strains isolated in the US, Postic *et al.* (1998) identified a new specie, *B. bissettii* sp. nov., and a cluster of other related but distinct strains, which require further characterization (e.g. the disease status of these isolates is unknown). *Borrelia corieaceae* isolate, is an example of an uncharacterized strain, that was isolated from the *Ornithodoros corieaceaous* tick and may be more closely related to the relapsing-fever pathogen than Lyme disease spirochetes.

The ability to isolate and culture the etiological agent of Lyme disease is essential in both research and clinical environments. For instance, the ability to produce a viable culture of *B. burgdorferi* in vitro has facilitated production of spirochetes' antigens, which allow detection of Lyme pathogens in clinical specimens (Steere *et al.* 1983). In addition, isolation of the pathogen from infected animals and ticks has been effective for defining endemic Lyme disease areas (Anderson *et al.* 1985 and 1989; Callister 1988).

Attempts to culture *Borrelia* go back to the early 20th century, when Kligler and Robertson (1922) defined the conditions for maintenance and growth of the relapsing fever pathogen in derivatives of Noguchi's medium. Today, variations of the complex Barbour-Stoenner-Kelly (BSK) growth media are traditionally used for in vitro cultivation of *Borrelia* spirochetes (Barbour 1984, Norris *et al.* 1997, Picken *et al.* 1997).

BSK II and standardized BSK H are two media of interest in the following study (Barbour 1984, Pollack *et al.* 1993). Since BSK II medium is not available commercially, it must be prepared in the individual laboratories from several components. Consequently, batch variations in media can influence growth kinetics, morphology, and antigenic characteristics of Lyme disease spirochetes (Callister *et al.* 1990). The latter medium, BSK H, was developed in order to create a source of readily available standardized medium. Table 1 describes the specific differences in the formulation of two media.

Although both BSK II and BSK H media are routinely used to grow *B. burgdorferi* in vitro, each medium shows some selectivity for specific isolates (Kleinjan 1999, pers. comm.). The purpose of this study was to compare two growth media, BSK II and BSK H, based on their ability to support the growth of three *Borrelia* isolates: *B. burgdorferi* sensu stricto, *B. bissettii* sp. nov., and *B. corieaceae* isolate; and to compare the growth kinetics of high-passage (P10) and low-passage (P7) *B. bissettii* CA389 isolates in order to evaluate if adaptation to cultural media facilitates faster growth in the isolates. In addition, the effects

of increased media viscosity on bacterial growth, via addition of gelatin to the media, were studied.

BSK H*		BSK II (CMRL)	
Ingredient	mg	Ingredients	mg
Arginine……	57	Arginine……..	70
Citric acid (tetrasodium)…….	700	Sodium citrate……..	800
2'-Deoxycytidine-HCL……..	11.6	2'-Deoxycytidine…..……..	10
Flavin adenine dinucleotide……	0.106	Flavin adenine dinucleotide…..	1.0
Gelatin……	**0**	**Gelatin**……..	**14000**
Magnesium Sulfate…….	97.69	Magnesium Sulfate…….	200
Sodium Acetate……	50	Sodium Acetate……...	83
Sodium Glucaronate…..……	3.88	Sodium Glucaronate……...	4.2
Sodium Phosphate monobasic..	122	Sodium Phosphate monobasic..	140

* BSK H contains endotoxin-tested tissue culture grade water

Table 1. Comparison of ingredients that differ between BSK H and BSK II media.

Materials and Methods

Bacterial strains and Growth Kinetics The designations, passage levels, and origins of the *Borrelia* strains used in this study are given in Table 2.

In order to evaluate the growth supporting capabilities of BSK II and BSK H media, four replicate cultures of each *Borrelia* isolates were set up in both media and incubated for ten days. To culture the spirochetes, 0.5ml of isolates in glycerol stock solution were added to 5 ml of each medium in a sterile culture tube and sealed to maintain the gas balance. The tubes were incubated in the dark at 34° C. The spirochetes were sampled a total of five times, once every other day, during the ten-day incubation period. Each sample was taken aseptically by removing 0.025 ml of medium with a sterile pipette. Initial density of spirochetes within the glycerol stock solution and the number of organisms per milliliter of growth media was determined using Petroff-Hausser counting chamber. Briefly, a drop of the sample was placed on the counting chamber (Kleinjan 1999, pers. comm.). The slide was left undisturbed for five minutes to allow the spirochetes to settle. After that time it was examined at 400X using dark field microscopy (DMF); the number of organisms within five clearly marked squares were counted and used to calculate the number of the spirochetes per milliliter of media.

Genospecie	Strain	No. of passages	Source and geographic location in the U.S.
B. burgdorferi	CA4	6	*I. pacificus* nymph / Cloverdale, CA
B. bissettii	CA389	7	*I. pacificus* nymph / Tilden Park, CA
	CA389	10	*I. pacificus* nymph / Tilden Park, CA
B. coriaceae	CA435	6	*O. corieaceous* adult / Hopland, CA

Table 2.
Spirochetes used
to evaluate media

Gelatin Experiment In order to determine an optimal gelatin concentration for the growth of *Borrelia* strains, the growth kinetics of the bacteria was evaluated at different gelatin concentrations using BSK II and BSK H as the basic media. Two concentrations of the thickening agent (14 g/L and 7g/L) were tested with *B. burgdorferi* CA4-p6, *B. bissettii* CA389-p7, and *B. corieaceae* CA435-p6. Four replicates of each culture were run for 8 days, along with the controls lacking in gelatin. The number of organisms was counted on days 4 and 8 of the incubation period with a Petroff-Hausser counting chamber according to procedures described above.

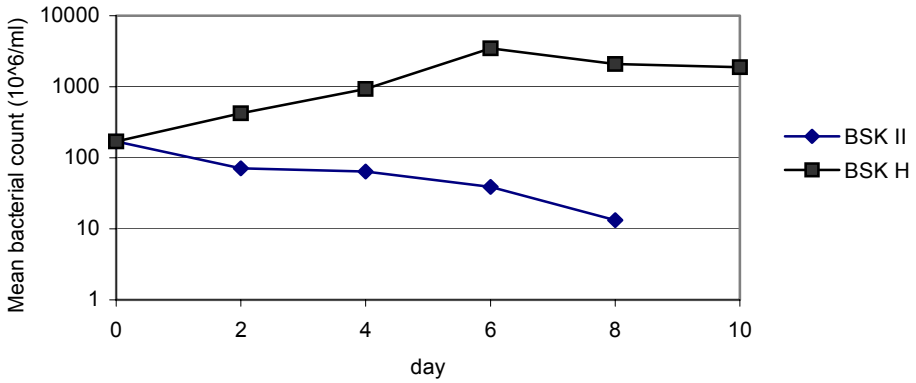
Statistical analysis Mean bacterial counts were calculated based on four replicate cultures. Student's t-test was used to determine whether growth of each strain examined is significantly different ($\alpha<.05$) when cultured on BSK II and BSK H.

Results

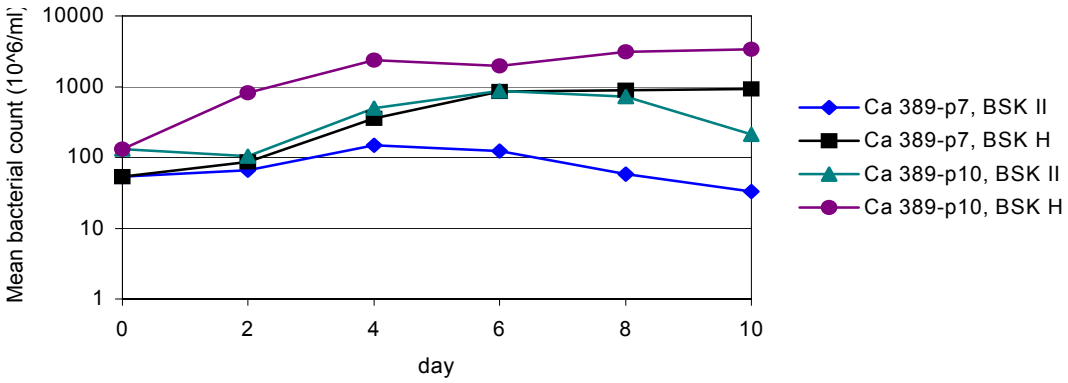
In vitro growth of *B. burdorferi* CA4 Comparison of *B. burgdorferi* growth curves in BSK H and BSK II media indicates that BSK H has superior growth supporting capabilities (Fig. 2a). Based on criteria such as coiling and degree of motility, the spirochetes appeared healthier and grew significantly faster in BSK H media ($p\leq0.0001$). Starting from an initial concentration of about $2x10^8$/ml, *B. burgdorferi* CA4 grew steadily to cell densities of about $3x10^9$/ml in BSK H. In contrast, *B. burgdorferi* CA4 growth curve in BSK II media shows a decline in total cell population between each sampling instance with final cell yield less than 10^7 cells/ml.

To determine whether or not increased viscosity aids the growth of spirochetes, gelatin was added to the BSK H and BSK II media to standardized final gelatin concentration

of 7g/ml and 14g/ml. Cultivation of *B. burdorferi* in BSK H media resulted in comparable growth at both concentration levels (Fig. 3a). However, in the case of control (no gelatin), the



a. *B. burgdorferi* CA4-P6



b. *B. bissettii* CA389-p7 and *B. bissettii* CA 389-

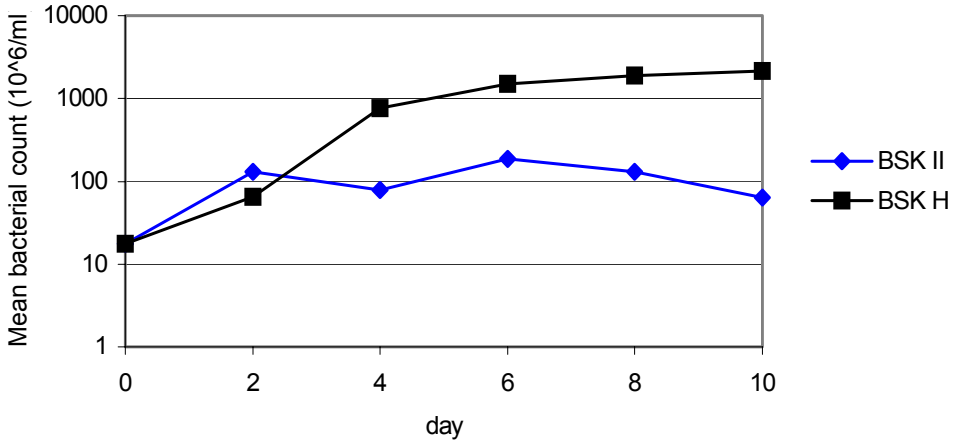
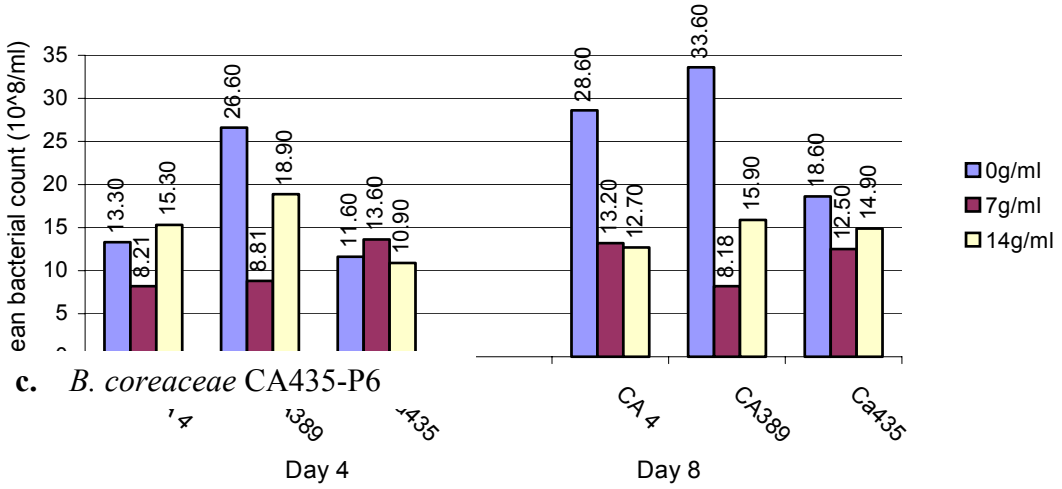
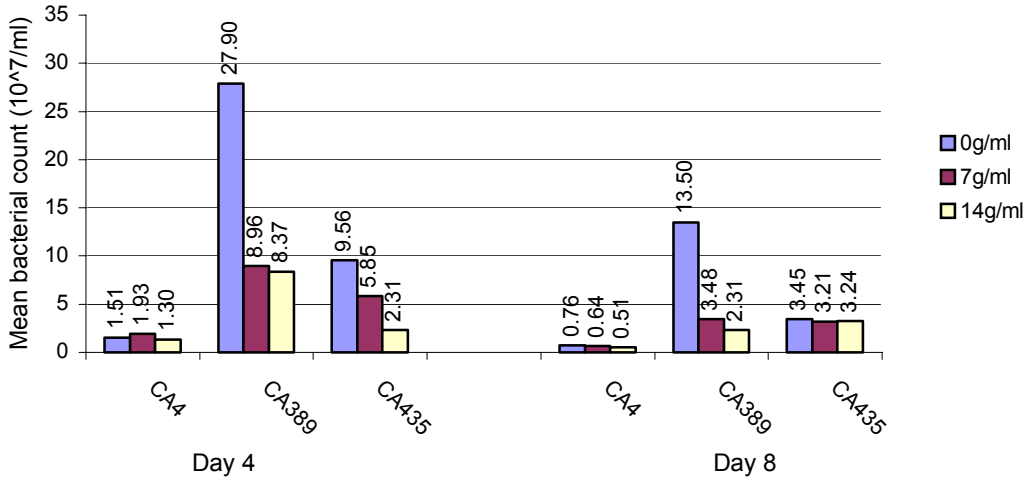


Figure 2. Growth curves of *B. burgdorferi* CA4-p6, *B. bissettii* CA389-p7 and *B. bissettii* CA 389-p10, and *B. coreaceae* CA435-p6 in BSK II and BSK H media.



a. Isolates growth at different gelatin concentrations in BSK H medium



b. Isolates growth at different gelatin concentrations in BSK II

Figure 3. *B. burgdorferi* CA4-p6, *B. bissettii* CA389-p7, and *B. corieaceae* CA435-p6 growth at different gelatin concentrations in BSK H and BSK II media. Note the magnitude difference in bacterial counts (y-axes) between BSK H and BSK II media.

cell concentration was significantly greater ($p \leq 0.001$) on day eight of incubation period. Similar trends were noticed in BSK II (Fig. 3b), where the spirochetes reached significantly higher densities in the media lacking in gelatin by day eight after inoculation.

In vitro growth of *B. bissettii* CA389 Both low passage (p-7) *B. bissettii* CA389 and high passage (p-10) *B. bissettii* CA389 isolates produced essentially similar shape growth curves (Fig. 2b). According to Figure 2b, each isolate attained greater density in BSK H than in BSK II media within several hours of inoculation. In addition, the percent change in number of spirochetes during each sampling instance, as compared to the initial density of spirochetes, was significantly greater for *B. bissettii* CA389 p-10 than for *B. bissettii* CA389 p-7 in both BSK H and BSK II media (Student's t-test; $p \leq 0.0001$ and $p \leq 0.001$ respectively).

Examination of effects of increased media viscosity on growth of *B. bissettii* CA389-P7 revealed that presence of gelatin inhibited cell growth in both BSK H and BSK II media (Fig. 3a, b).

In vitro growth of *B. corieaceae* CA435 *B. corieaceae* CA435 growth curves for BSK H and BSK II media (Fig. 2c) illustrate that the isolate grew significantly faster in BSK H media ($p \leq 0.00001$). The shape of BSK II growth curve shows a quick growth spurt within the first two days of inoculation followed by stationary phase at nearly constant cell concentration of approximately 10^8 cells/ml. The spirochetes incubated in BSK H exhibited exponential growth pattern with total cell yield of approximately $3x10^9$ cells/ml.

Although presence of gelatin had no significant effect on the growth of *B. corieaceae* in BSK H media, from Figure 3b it is evident that gelatin inhibited growth of the spirochetes in BSK II ($p \leq .01$).

Discussion

Isolation and cultivation of *Borreliae* isolates is performed generally in modified Barbour-Stoenner-Kelly (BSK) growth media in a microaerophilic environment. This study evaluated growth of *B. burgdorferi* sensu stricto CA4 isolate, a low passage (P7) and a high passage (P10) isolates of *B. bissettii* sp. nov. CA389, and *B. corieaceae* CA435 isolate in BSK H and BSK II media. The results demonstrate a significant difference between the growth of the isolates in BSK H verses BSK II media and indicate that BSK H has improved growth-supporting capabilities.

Comparison of low passage (p-7) *B. bissettii* CA389 and high passage (p-10) *B. bissettii* CA389 growth indicates that the high passage isolate grows significantly faster in both media. This correlation between the growth rate and the number of passages in the

cultural media demonstrates the adaptation phenomenon that is found in many pathogenic bacteria (Melton and Weiss 1989; Ernst et all. 1990). In many cases bacteria respond and adapt to their hosts by sensing changes in the environment. These environmental signals may include change in nutrient availability and temperature, osmolarity, pH, concentration of oxygen (O_2) and carbon dioxide(CO_2) (Mekalanos 1992). Similar environmental cues may influence bacterial growth in vitro; and the length of time a spirochete has been maintained in culture influences its success in utilizing any growth media.

Since *Borreliae* are microaerophilic (Barbour and Hayes 1986; Kelly 1971), the environment with little gaseous oxygen should be maintained to obtain a viable culture. In this study such environment was established by filling culture tubes with media to 60% capacity and capping tightly in ambient air. The cap was used to maintain the gas balance within the medium; however, a confounding factor was introduced when the sample was taken.

In order to delineate the factors that inhibit growth of Borrelia in BSK II medium, the ingredients within BSK H and BSK II media were compared. Table 1 summarizes the differences between two formulations. Gelatin and flavin adenine dinucleotide (FAD) are the two compounds that vary by greatest amounts in BSK H and BSK II media. FAD was not tested in this experiment because fermentation is the principal metabolic pathway for *Borrelia* (Fulton et all. 1960) and compound has no apparent role in the media used to culture the spirochetes--it acts as an electron carrier in Krebs cycle and oxidative phosphorylation steps of cellular respiration (Campbell 1996). Conversely, according to Barbour *et al.* (1986), spirochetes generally prefer a viscous environment, thus, gelatin may be an important component of the medium.

The effect of gelatin on the growth of *Borrelia* spirochetes was evaluated in the second part of this study. The results indicated that gelatin inhibits growth of *B. burgdorferi* CA4 and *B. bissettii* CA389 in BSK H and BSK II media and has no significant effect on the growth rate of *B. corieaceae* CA435 in BSK H. This suggests that various genospecies of *Borrelia* respond differently to a given environmental factors (e.g. media's viscosity), emphasizing the difficulty in cultivating these spirochetes.

Norris *et al.* (1997) have shown that process of culturing *Borrelia* spirochetes in BSK H media isolates a limited number of the genospecies circulating in a population. Other

studies have shown that various culture conditions, such as, osmotic strength, dissolved oxygen and carbon dioxide concentration, and temperature of the growth media can influence retention and/or expression of the infective phenotype (Elias *et al.* 1998; Austin 1993). According to this study gelatin concentration or medium's viscosity may also play an important role in growth of *Borrelia*; however, a greater number of isolates should be evaluated in order to determine the effects of gelatin on the individual genospecies of *Borrelia* and the optimal viscosity of the growth media.

The results of this study suggest that BSK H has superior growth supporting capabilities. For that reason, direct comparisons of research results using spirochetes maintained in BSK H and BSK II media are inadequate, and reevaluation of the studies that used BSK II as the growth media is warranted.

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