Growth, cysts and kinetics of *Borrelia garinii* (Spirochaetales: Spirochaetacea) in different culture media

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The aim of the present paper was to evaluate cyst formation and growth parameters of Borrelia garinii *in a range of media differing in formulation and cost. A qualitative assessment of morphology and motility of* B. garinii *was conducted. All media were prepared aseptically and used in test tubes or Petri dishes. For each medium, the initial spirochete concentration was standardized to 10^3 spirochets/mL. The following culture media were suitable to grow* B. garinii*: Barbour-Stoenner-Kelly, brain heart infusion and PMR. Growth was minimal at six weeks post-inoculation and maximum spirochete density was observed between 9-12 weeks. Often, the cultures developed cysts of different sizes, isolated or in groups, with a spiraled portion of variable sizes, mainly in unfavorable culture media. Brazilian Lyme disease-like illness, also known as Baggio-Yoshinari syndrome (BYS), is a new and interesting emerging tick-borne disease, caused by* Borrelia burgdorferi sensu lato *spirochetes, only during its cystic forms. It has been assumed that the peculiar clinical and laboratory features of BYS are consequential to the absence of a human sucker* Ixodes ricinus *complex tick at risk areas in Brazil, supporting the concept that the borrelia phenotypic expression pattern is modified as it is transmitted through the host.*

Key words: culture media - *Borrelia garinii* - spirochaetaceae - kinetic growth - cysts

Borrelia garinii, a bacterium of the Spirochaetaceae family, is an etiologic agent of Lyme disease (LD) for humans in Europe and Asia (Baranton et al. 1992). In Europe, *B. garinii* is transmitted to rodents, birds and humans by ticks of the *Ixodes ricinus* complex. Olsen et al. (1995) demonstrated the role of migratory birds as vectors that disperse ticks and discussed the potential for the occurrence of silent *Borrelia* cycles in different continents, including the southern hemisphere.

The discovery of culture media to support the growth *Borrelia* species has improved the knowledge about the morphological characteristics of the bacterium and colonies and has provided a means of studying the different antigenic structures of the microorganism (Barbour 1984). Many modified culture media have been evaluated, aimed at achieving the best spirochete growing conditions to produce a sufficient amount of cell mass for immunodiagnostics, the production of immunogens and for the culture of clinical specimens (Preac-Mursic et al. 1991, Pollack et al. 1993, Gruntar et al. 2001).

Oliveira et al. (2004) studied the growth behavior of *Borrelia burgdorferi* using eight different culture media and observed that spirochete growth occurred from week four and reached a plateau between 8-12 weeks. In addition, the authors observed that cystic forms of the pathogen developed in all media tested. Rodríguez et al. (2007), after replacing CRML 1066 with 199 medium, compared the growth of different strains of *Borrelia* in relation to the modified Barbour-Stoenner-Kelly (BSK)-H medium with added amino acids and antibiotics and indicated that the modified medium could function as an alternative growth media for the cultivation of *Borrelia* strains.

The spirochetes that cause LD grow at 34ºC, in selective or non-selective culture media and are visualized using dark-field or phase contrast microscopy. De Martino et al. (2006) have reported the growth of *B. burgdorferi*, *B. garinii* and *Borrelia afzelii* in BSK solid medium, where they compared two incubation methods, one with 3% CO_2 and the other in anaerobic conditions.

Nutrient-rich media and the prolonged incubation required to cultivate *Borrelia* species not only increase the risk of contamination with other microorganisms but also are costly. Therefore, this paper aimed to assess the feasibility of using modified and less expensive culture media to grow *B. garinii* and to evaluate cyst formation.

Samples of *B. garinii* (strain IB29), originally provided by Dr. Arno Schönberg, from Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany, were stored in Nunc™ tubes containing BSK medium with 8% sterile glycerol and kept frozen in liquid nitrogen at the University of São Paulo Rheumatology Laboratory (LIM-17 HCFMUSP). The spirochete samples have been maintained for more than 10 years in the laboratory and have been cultured in BSK medium for more than 90 passages.

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The following culture media BSK, PMR and brain heart infusion (BHI) were used to grow the spirochete, as previously described by Preac-Mursic et al. (1991). In addition to these, media for growing flagellated bacteria also included Brucella Broth, Tarozzi, CTB and Dubos Broth, each with and without antibiotics and at different pH concentrations.

All culture media were prepared aseptically and stored in 10 mL test tubes or in 50 x 20 mm Petri dishes at 34ºC. For each medium sample, the initial spirochete concentration was standardized to 10^3 spirochetes/mL. At least four replicate plates were used to inoculate each medium tested. Spirochete growth was assessed by dark field microscopy that varied according to the stage of the experiment. Spirochetes were counted in a Neubauer™ chamber, using phase contrast or dark field microscopy at 250X magnification. Bacterial counts were performed daily for two weeks. After this, bacterial growth was evaluated twice per week to week 14.

Of the liquid or solid culture media tested, BSK, BHI and PMR promoted reasonable growth rates of *B. garinii* after six weeks. In these media, *B. garinii* started to grow during the 6th week; growth reached a plateau between weeks nine and 12, whereupon the culture medium became exhausted of nutrients (Fig. 1). No growth was observed in Tarozzi Broth and growth in the Brucella Broth, CTB and Dubos Broth was insignificant.

Borrelia spp are difficult to culture in vitro and many ingredients have been added to the medium to improve bacterial growth, including rabbit serum, bovine serum albumin (BSA), peptones, sodium citrate pyruvate and N-acetylglucosamine. Callister et al. (1990) demonstrated that addition of Fraction V, BSA, improved *B. burgdorferi* growth, especially when the number of spirochetes in the medium was low. The authors tested six different batches of commercially available BSA, obtained from three manufacturers (Armour, Sigma and Gibco) and verified that the morphology of the bacteria, mainly its motility and granule formation, varied greatly according to selection of ingredients. Furthermore, the supply source of albumin Fraction V interfered with *B. burgdorferi* protein expression, based on indirect immune fluorescence assays.

Pollack et al. (1993) concluded that protein components, such as BSA, including its origin and quality, were critical factors that influenced the dynamics of spirochetes growth. According to Johnson and Rogers (1964), 5-fluorouracil, a uracil analogue, plays an active role inhibiting growth of several species of bacteria, including other contaminating microorganisms.

Aberrant or cystic forms of spirochetes were seen primarily in poor growth media or when the media was close to nutrient exhaustion (Fig. 2). Oliveira et al. (2004) identified aberrant forms of spirochetes while studying the growth of *B. burgdorferi* and demonstrated that the appearance of unusual *Borrelia* cysts is a common feature to the *B. burgdorferi sensu lato* complex. According to Brorson and Brorson (1997), adverse conditions such as the presence of antibiotics, stimulate the appearance of cysts, which may be a consequence of the microorganisms ability to survive in deficient or extreme conditions. Cystic forms and their ability to convert into normal motile spirochetes have already been demonstrated in the *B. burgdorferi s. l.* complex (Gruntar et al. 2001). These authors also showed the capacity of the *B. garinii* cysts to reconvert into motile spirochetes in vitro and in vivo with unexpected resistance to adverse environmental conditions. Gruntar et al. (2001) also proposed that *Borrelia* spirochetes are not fragile and delicate microorganism as might be expected from their difficult isolation. According to Murgia and Cinco (2004), the phenomenon of conversion to cysts by *B. burgdorferi* provides a limited survival potential. This short-term survival, however, provides an additional opportunity to overcome unfavorable environmental conditions.

Antibiotic efficiency requires active bacterial metabolism. Cystic form microorganisms are generally not pathogenic, but are resistant to antibiotic treatment because most of these drugs act at the bacteria cell wall.

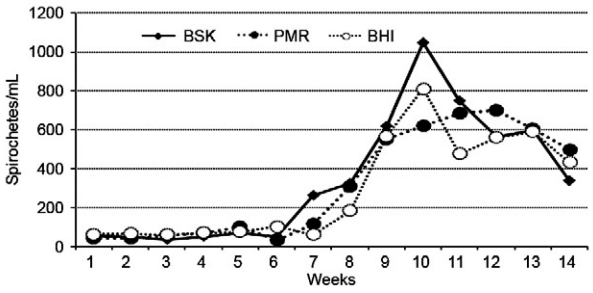

Fig. 1: kinetic of *Borrelia garinii* (strain IB 29) growth in Barbour-Stoenner-Kelly (BSK), PMR and brain heart infusion (BHI) culture media expressed in weeks.

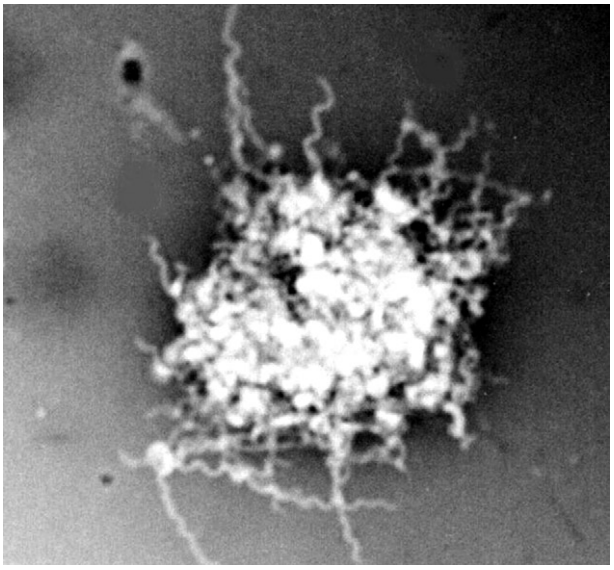

Fig. 2: growth of the cysts forms of *Borrelia garinii* (strain IB 29) on PMR media. L-form with thin filaments extending in various directions and often ending in swollen knobs. Fifteen weeks culture, 34ºC, nigrosine, light microscope, 1000X.

According to Brorson and Brorson (1997), this phenomenon explains why some LD patients are difficult to treat at the later stage of the disease.

Furthermore, Brazilian LD-like illness (BLDLI) or Baggio Yoshinari syndrome (BYS) is a tick-borne disease which reproduces most of the symptoms observed in LD except for the high frequency of recurrent clinical symptoms, autoimmune features and the need for prolonged antibiotic therapy (Yoshinari et al. 1997). Recent unpublished, molecular biology studies at the LIM-17 HCFMUSP, indicate that the etiological agent of BYS belongs to the *B. burgdorferi sensu lato* complex.

Since *B. burgdorferi* has never been isolated in Brazil and based on studies by Mantovani et al. (2007), we propose that BLDLI is an emerging and unique disease. It is caused by this spirochete at its atypical morphologic stage (cystic form), which explains the appearance of the particular clinical and laboratory features observed in Brazilian patients. A possible explanation for the emergence of atypical morphological borrelias in Brazil is the absence of the human blood suckers *I. ricinus* complex ticks within BYS risk areas.

For many years, different culture media have been tested to grow, without success, the cystic forms of spirochetes found in Brazilian patients. In our studies reported here, we attempted to find a suitable growth culture medium for *B. burgdorferi sensu lato*. Surprisingly, we observed the presence of cysts of the same morphology as found in BYS patients, after testing with different culture media; thus, demonstrating that *Borrelia* spp are very unstable microorganisms and require specific growth factors to maintain the complete motile spiraled morphology.

This paper reinforces the concept that *B. burgdorferi s. l.* spirochetes are very sensitive to environmental conditions, supporting the idea that the existence of different vertebrate and invertebrate hosts in Brazil can explain the occurrence of atypical *B. burgdorferi* in this country. It is also possible that complete mobile spiraled borrelias do not exist in Brazil because the typical spirochete was never recovered from BYS patients after their biological samples were seeded in different culture media. In conclusion, our results lead us to propose that BLDLI may be an emergent clinical disorder distinctive to Brazil.

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