

Evaluation of a modified culture medium for *Borrelia burgdorferi* sensu lato

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The aim of the present study was to assess the possible use of a modified medium, prepared in the laboratory using the constituents of Barbour-Stonner-Kelly (BSK) medium and medium 199 as base, for the culture of Borrelia strains, comparing the growth of individual strains in this medium and in the BSK-H medium, and the protein profile and antigenic characteristics of Borrelia proteins expressed in these media. A qualitative evaluation of growth of Borrelia species was made with acceptable results (morphology and motility), but during a quantitative evaluation using the three main genospecies of Borrelia, the better results were obtained with a B. burgdorferi sensu stricto strain. The modified medium did not enable the growth of a B. afzelii strain. The protein profile and antigenic characteristic of the expressed proteins in the modified medium were studied with satisfactory results. These results suggest the modified medium as an alternative for the cultivation of Borrelia strains, with some limitations, in poorly-resourced laboratories.

Key words: *Borrelia* - new medium - BSK-H medium - proteins

Lyme borreliosis represents the most common vector-borne zoonotic disease in the Northern hemisphere. It is caused by several closely related *Borrelia* species that are collectively referred to as *Borrelia burgdorferi* s.l. The human infection is caused primarily by three pathogenic genospecies, *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* (Steere 2006). In North America, *B. burgdorferi* s.s. is the sole cause of the infection, while in Europe the three genospecies can cause the disease, and in Asia the latter two species. In addition, nine other closely related species have been identified, but only *B. lusitanae*, *B. valaisiana* and *B. spielmani* cause human infection. These organisms live in nature in enzootic cycles involving different species of ticks, mainly *Ixodes* complex (Jouda et al. 2004, Derdákova & Lencáková 2005, Grubhoffer et al. 2005, Evison et al. 2006, Steere 2006).

Borrelia species can be isolated from different clinical samples, such as skin, blood, and cerebrospinal fluid of patients with early as well as chronic infection indicating their ability to infect different organ and tissue types and to maintain the infection. Isolation of *Borrelia* species from clinical material is a golden standard for confirming borreliosis infection, although it is quite a demanding and long-lasting procedure with limited sensibility (Ruzic-Sabljić & Strle 2004, Ruzic-Sabljić et al. 2006). Different culture media have been evaluated

and used for cultivation, but most frequently Barbour-Stonner-Kelly (BSK) medium (Gorelova & Shcherbakov 1991, Marques et al. 2000, Ruzic-Sabljić & Strle 2004).

The aim of the present work was to assess the possible use of a modified medium for the culture of *Borrelia* strains comparing the growth of individual strains of *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* in this medium and in medium BSK-H prepared according to Sinsky and Piesman (1989). We also analyzed the protein profile and antigenic characteristics of *Borrelia* proteins expressed in the modified medium.

The modified medium was prepared using the constituents of BSK medium and medium 199 as base in substitution of medium CMRL 1066, following the original recipe (Barbour 1984). Briefly, 5 g of neopeptone (Difco Laboratories, USA), 50 g of bovine serum albumin (BSA) (fraction V) (Sigma Chemical Co, USA), 6 g of N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (BDH Laboratory Supplies Poole, England), 0.7 g of bi-hydrated tri-sodium citrate (BDH), 5 g of D(+)-anhydrous glucose (BDH), 0.8 g of sodium pyruvate (BDH), 0.4 g of N-acetyl-D-glucosamine (Sigma), 2.2 g of sodium bicarbonate (Sigma), and 2.53 g of TC yeastolate (Difco) were added to 900 ml of milli-Q water. The solution was slowly stirred until total suspension of the ingredients, then 100 ml of medium 199 (10X) with L-glutamine (GibcoBRL, USA) was added. The medium was adjusted to pH 7.6 with 1N NaOH, and 64 ml of heat-inactivated rabbit serum (in house-preparation) was added, then it was sterilized by filtration through a 1.2-0.45-0.22 µm filters (Millipore Corp). Finally, 200 ml of gelatin 7% (Oxoid, England) sterilized by autoclave was added. After preparation, 6.0 ml aliquots of medium were dispensed into polystyrene culture tubes (13 by 100 mm) (PolyLabo, France).

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BSK-H medium was prepared in parallel using BSK-H medium (Sigma), heat-inactivated rabbit serum (Sigma) and gelatin (Difco). Amino acids and antibiotics were added according to Sinsky and Piesman (1989). The medium was aliquoted as mentioned above.

A qualitative evaluation of *Borrelia* growth was carried out using *B. garinii* (NE83) and *B. valaisiana* (NE4555) obtained from free-living *I. ricinus* ticks collected in Switzerland. The number of spirochetes per milliliter was determined with a counting chamber (Helber's cells). Identical volumes with the same concentration of each strain were added to duplicate tubes containing the media and incubated for 10 days at 35°C, and motile and uniformly refractile spirochetes were observed in both media by dark-field microscopy, but the cellular concentration was lightly superior in BSK-H medium. Later, a quantitative evaluation for comparing *Borrelia* growth was done. Three low-passages (2 passages) *B. burgdorferi s.l.* strains were randomly selected from the collection of the isolates of the laboratory of Eco-Epidemiology of Parasites at the Institute of Biology of the University of Neuchâtel (Switzerland). *B. burgdorferi s.s.* (NE4502), *B. garinii* (NE4546) and *B. afzelii* (NE4505) were obtained from free-living *I. ricinus* ticks collected in Switzerland. Strains were identified to species level by polymerase chain reaction/restriction fragment length polymorphism analysis as previously described (Postic et al. 1994) or by reverse line blot (Poupon et al. 2006). Inoculum volumes of each strain were determined, using the counting chamber, for a final concentration in the fresh media of 1.5×10^4 spirochetes/ml. The media were inoculated simultaneously, 10 replicas of each strain were made for the modified medium and five replicas for the BSK-H medium. All the media were incubated to 34°C. To assess the growth of individual strains in BSK-H and modified medium, the number of cells was determined 2, 4, 7, 10, 14, and 23 days after inoculation using the counting chamber and curves of growth were done (Fig. 1) with the mean values of concentration of each strain. Dark field microscopy was used in order to detect any contamination in the media and to check the morphology and motility of borrelias. No differences in the latter aspects were observed between the media.

The logarithmic phase for *B. burgdorferi s.s.* in the modified medium was slower than in BSK-H, but the concentrations of spirochetes during the stationary phase in both media were similar, and the number of borrelias in the decline phase was superior in the modified medium.

The results differed for the other *Borrelia* species. In the modified medium the number of *B. garinii* cells was lower than in BSK-H until day 14, the logarithmic phase was very slow and the stationary phase was not clearly observed, and the decline phase was similar in both. The multiplication of *B. afzelii* strain in the modified medium was not possible; we only observed some borrelias with reduced motility. The number of cells at the beginning of culture was relatively low, but we used this one because it enables a quantitative approach to the number of spirochetes in the samples.

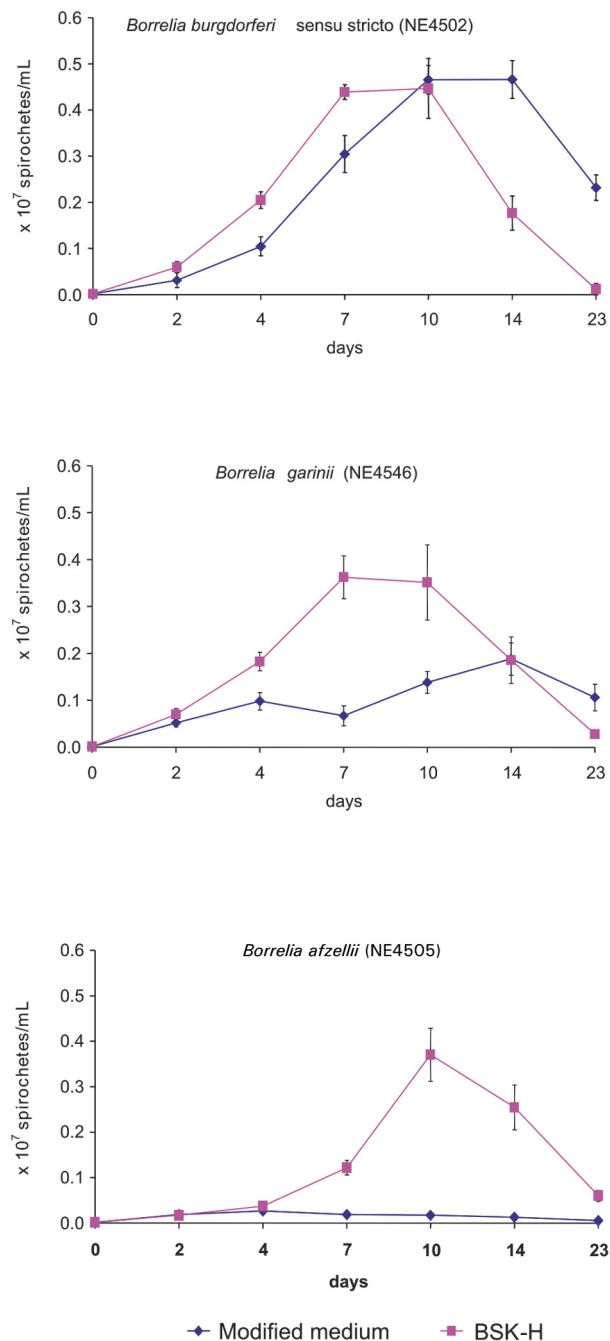


Fig. 1: growth curves of *Borrelia* strains in different media.

The growth of *Borrelia* strains differed also in the BSK-H medium and not only in the modified medium prepared with medium 199. Strains from different *Borrelia* species grew differently, maybe due to the fastidious nature of the organism and changes that *Borrelia* must undergo to adapt the transition from living biological material to an artificial medium, since the *Borrelia* strains had received only two passages starting from the primary isolation.

It is known that borrelias are microorganisms fastidious for growth in defined chemical media, and changes of batches and/or commercial house of medium constituents affect the ability of the medium to growth *B. burgdorferi s.l.* It has been demonstrated, for example, that the fraction V BSA can affect the recovery of low numbers of borrelias (Callister et al. 1990).

Medium ingredients and other factors including temperature of incubation, pH of the medium, and capacity of particular borrelial species can also influence on the borrelial growth (Ruzic-Sabljić & Strle 2004). In this study we only modified the composition of medium. When comparing the CMRL 1066 medium (GibcoBRL, USA) with medium 199 (GibcoBRL, USA), no significant differences were found in relation to inorganic salts, amino acids and vitamins composition; however, the concentration of amino acids in medium 199 was higher and the chemical composition of other non-essential components for *Borrelia* differed from one medium to the other.

Another purpose of the present work was to analyze the protein profile of a *Borrelia* strain in the modified medium and to know the antigenic properties of expressed proteins. A culture of *B. garinii* (NE83) in the modified medium during 10 days at 34°C was made. The cells were harvested, washed and lysed in Laemeli buffer containing Tris-HCl 0.5M buffer, sodium dodecyl sulfate, 2-mercaptoethanol, glycerol and bromophenol blue, and finally the spirochetes were boiled for 3 min. Electrophoresis was performed in polyacrylamide gels (12.8 % acrylamide running gel and 6 % acrylamide stacking gel) as described by Laemeli (1970). The gels were transferred to nitrocellulose membranes, one membrane was stained with colloidal gold and the other was used for a Western blot-IgG (home-made) with a human positive control serum to Lyme Borreliosis by ELISA (IgM/IgG) (BioMérieux), and Western blot-IgG with *B. garinii*, *B. burgdorferi s.s.* and *B. afzelii* antigens. The results are shown in Fig. 2.

The major *Borrelia* proteins (specific and non-specific) were expressed by *B. garinii* in this modified medium according to colloidal gold stain picture. The results of Western-blot analysis of whole-cell lysates of *B. garinii* probed with the human immune serum demonstrated that a large number of expressed proteins are antigenic, which did not change when the *Borrelia* grew in different media. However, some differences in the region of 20-25 kDa proteins (presumably OspC) were observed. Considerable antigenic variations have been documented for *Borrelia* strains; besides the adaptive mechanisms of the spirochetes that influence protein expression, each strain has its own potential to express a particular protein (Wilske et al. 1996, Ruzic-Sabljić et al. 2001). According to these results, the antigen obtained could be used in a Western-blot for the diagnosis of Lyme borreliosis in humans.

To be useful for laboratory purposes, the medium must be stable, available and efficacious in supporting the growth of all human pathogenic *Borrelia* strains (Ruzic-Sabljić & Strle 2004), including *B. lusitanae*, *B. valaisiana* and *B. spielmani*; this latter element was

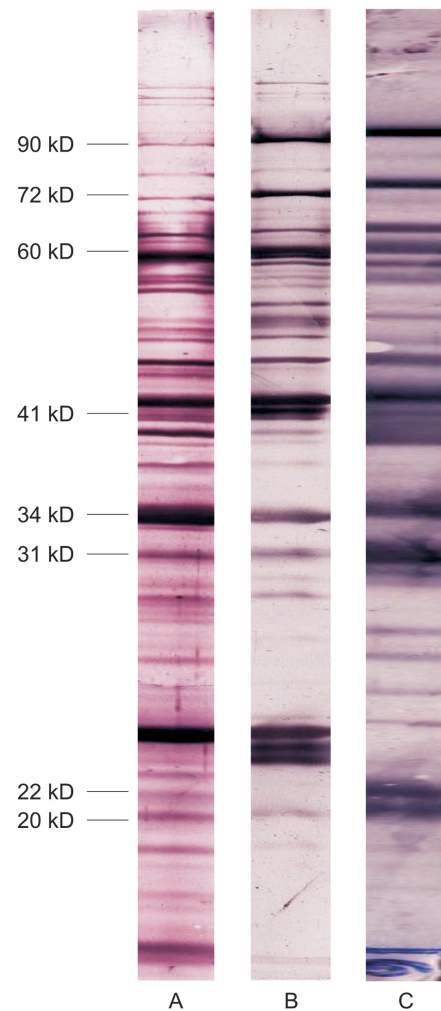


Fig. 2: whole-cell lysates of *Borrelia garinii* (NE 83) from different media. A: lysate from the modified medium with colloidal gold stain. B: western blot of lysate from the modified medium. C: western blot of lysate from BSK-H medium.

not well defined in this work, because the main limitation of the present study was the low number of used strains. Further works with a larger number of strains is needed to compare the results of this study on the differences in the growth of *Borrelia* species in the modified and BSK-H medium (Sinsky & Piesman 1989).

The modified medium evaluated in the present study could be an alternative for the culture of *B. burgdorferi s.l.* strains taking into account its limitation for some strains in Latin-American and Caribbean countries with poorly-resourced laboratories, although there are not much published papers about the presence of *Borrelia* genospecies in this region, only serological evidences of Lyme disease have been published (Ciceroni et al. 1994, Abel et al. 2000, Rodríguez et al. 2004, G Gordillo et al. unpublished observations).

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