



RESEARCH ARTICLE

Evaluation of BSK-H Complete Medium Supplemented with Rabbit Serum and Sodium Bicarbonate for the Growth of *Borrelia anserina*

Bilal Aslam, Iftikhar Hussain*, Muhammad Shahid Mahmood and Ahrar Khan¹

Institute of Microbiology, ¹Department of Pathology, University of Agriculture, Faisalabad, Pakistan

*Corresponding author: driftikharuaf@gmail.com

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ABSTRACT

The present study was conducted to compare the effect of 3 formulations Barbour-Stoenner-Kelly (BSK) medium on the growth of *Borrelia (B.) anserina*, the causative agent of avian borreliosis. Three different formulations of BSK medium (BSK-H, BSK-II and BSK-H complete medium) were prepared. For the isolation of *B. anserina*, Argas ticks were inoculated in all the three (BSK-H, BSK-II and BSK-H complete) formulations of BSK medium. All the samples were also observed for the impact of BSK medium on the growth (Generation time, Growth per hour, Specific growth rate) of *B. anserina*. Phase contrast microscopy was performed for the observation of viable *B. anserina* cells, and additional confirmation of all the isolates was done by performing indirect immunofluorescence assay and PCR. BSK-H complete medium supplemented with 6% rabbit serum and sodium bicarbonate was found best when compared to two other formulations of BSK medium with respect to the isolation, generation time and growth rate of *Borrelia* spirochetes.

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INTRODUCTION

Borrelia anserina, the causative agent of avian borreliosis causes heavy economic losses across the globe (Lisbôa *et al.*, 2009). *B. anserina* has been isolated from Argas ticks in Pakistan (Aslam *et al.*, 2012). Genus *Borrelia* belongs to family Spirochetaceae; spirochetes are fastidious organisms and require special care for their growth. Different nutrients present in the medium such as yeast extract, peptone, carbohydrates and amino acids facilitate the growth of the spirochetes (Wang *et al.*, 2004). Moreover the temperature is also very critical in maintaining the growth of the spirochetes; as *Borrelia* has to face a number of temperature conditions in three different environments, which includes the vector, host and the organ inside the host (Hubálek *et al.*, 1998) the optimal temperature for spirochetes ranges from 35-40°C. Temperature variation also has a vital impact on gene regulation in *Borrelia* during its growth in BSK medium (Pappas *et al.*, 2011). Most of the spirochetes are strict anaerobes with some exceptions and *Borrelia* is microaerophilic in nature. When spirochetes grow in the presence of oxygen, they produce a yellow pigmented growth in the medium due to the production of a carotenoid pigment (Paster and Dewhirst, 2000).

In the 1980s, *Borrelia* was recovered for the first time from Ixodes ticks using modified Kelly medium. Afterwards this modified medium was registered as Barbour-Stoenner-Kelly (BSK) medium and used to cultivate *Borrelia* frequently (Wang *et al.*, 2004). Different studies have been reported about the use of different modifications of BSK medium to grow *Borrelia* from different biological and geographical sources (Güner *et al.*, 2003; Ataliba *et al.*, 2007). Numerous modifications of BSK medium include BSK-II, BSK-H and modified Kelly medium, which have been used in different studies to cultivate *Borrelia* (Pollack *et al.*, 1993).

It has been established that BSK-H medium is the medium of choice for the cultivation of the *Borrelia*. Almost all of the modifications in BSK medium formulation contain some very vital ingredients like *N*-acetyl-glucosamine, yeast extract, amino acids, vitamins, nucleotides, and serum, but variations in several other chemical ingredients of BSK medium have also been reported (Pollack *et al.*, 1993). In the past, a medium having bovine serum albumin (BSA) and rabbit serum was standardized and named BSK-H medium (Wang *et al.*, 2004).

The proposed study was designed with a hypothesis that supplementation of BSK-H medium with rabbit serum

and Sodium bicarbonate has a significant impact on the cultivation of *B. anserina*. In the current study, we observed the impact of BSK-H complete medium on the growth of the *B. anserina* during its isolation and compared it with two other formulations of BSK medium (Sigma Aldrich, USA). A comprehensive knowledge of the isolation and growth of this infectious agent is very important from an ecological, epidemiological view point to formulate disease control strategies.

MATERIALS AND METHODS

Sample collection and processing: Adult Argas ticks were collected from different poultry farms, in small plastic tubes having 1% myostatin solution to avoid dehydration (Güner *et al.*, 2003). Three different formulations of BSK medium were used and dissected ticks were subjected to 5 ml of BSK-II medium, BSK-H medium, BSK-H complete (Sigma Aldrich, USA cat # B8291) medium, respectively, for the cultivation of the spirochetes. Afterwards, samples were incubated at 37°C (Barbour, 1984) under microaerophilic condition. Granular growth was observed daily up to 10 days post incubation.

Confirmation of the isolates: Isolates were confirmed with the help of dark field and phase contrast microscopy by observing the motile spirochetes (Aslam *et al.*, 2012). Additional confirmation of the isolates was done by performing the indirect immunofluorescence assay (IFA) following the procedure of Horta *et al.* (2004) with the help of FITC conjugated anti-Borrelia IgG antibodies (Bactrec™). For molecular identification, the isolates were subjected to polymerase chain reaction (PCR) by using specific primers against *fla B* gene with sequences: FP: 5'-ACA TAT TCA GAT GCA GAC AGA GGT-3', RP: 5'-GCA ATC ATA GCC ATT GCA GAT TGT-3' (Barbour *et al.*, 1996). Briefly, PCR master mix was made having a 2.4 µl of 25 mM of MgCl₂, 0.6 µl of 10 nM of dNTPs, 0.6 µl of 10 µM of both primers and 1 U of Taq DNA polymerase (Invitrogen). PCR master mix was subjected to a reaction of 30 repeated cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, final extension was done at 72°C for 10 min and at the end the reaction was terminated at 4°C. Afterwards, 0.8% agarose gel electrophoresis was performed to visualize the PCR products under the gel documentation system (Dolphin-Doc, Wealtec, USA).

Growth analysis: Growth of *B. anserina* was observed on daily basis from the 5th day post-inoculation to 10th day post-inoculation of the samples on the basis of granular growth in BSK-H medium and with the help of spectrophotometer. Furthermore, cells were counted with the help of hemocytometer/counting chamber (Rodríguez *et al.*, 2007) under phase contrast microscope after making the appropriate dilution *i.e.*, 1:10. By determining the following parameters a growth curve was constructed; these parameters were determined with the help of following formula (Heroldová *et al.*, 1998):

$$R (h^{-1}) = 1/\log_2 [(\log N_2 - \log N_1 / t)]$$

Where R (h⁻¹) = Growth per hour

N₁ = Bacteria present at an early stage in exponential phase

N₂ = Bacteria present at some time of exponential phase

t = time interval between N₁ and N₂

RESULTS

Isolation of borrelia: Typical granular growth was observed in all three kinds of BSK medium tubes. Although *B. anserina* was present in all kinds of BSK medium, passage time of Borrelia was found to be lesser (6 days) in BSK-H complete medium as compared to other formulations of BSK medium (8 days). Typical cells of *B. anserina* were observed under phase contrast and dark field microscope (Fig. 1). Subsequently, the isolates (n=15) reacted positively with FITC-conjugated anti-Borrelia antibodies, which confirmed the isolates. Since all the positive samples amplified (*fla B* gene) a PCR product of 750 bp (Fig. 2), that confirmed the isolates on molecular basis.

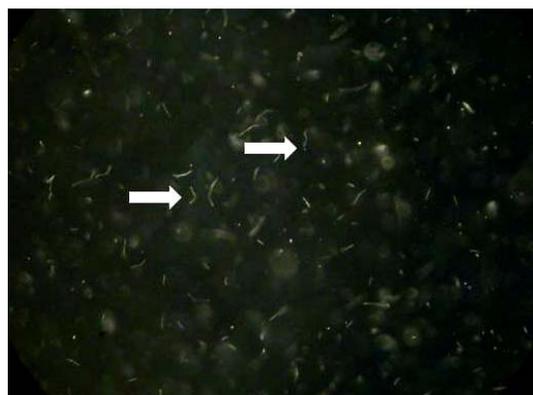


Fig. 1: Dark field microscopic image of *B. anserina* cells, showing typical wavy thread structure of Borrelial cell (100X).

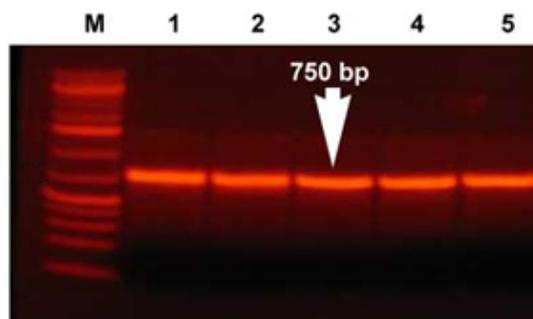


Fig. 2: Agarose gel electrophoresis: lanes (2-6) showed an amplified PCR product *fla B* gene of *B. anserina* (750 pb), whereas lane 1 & M showed positive control and marker, respectively.

Growth analysis: Growth curves of *B. anserina* in three different formulations of BSK medium are shown in Fig. 3. In both BSK-H medium and BSK-II medium, growth of Borrelia was found to be less as compared to BSK-H complete medium, as its passage time was observed less *i.e.*, 5 days whereas the passage time in other two medium were recorded up to 7 days. Among these three media, the shortest generation time (8 hours) was found for those samples, which were inoculated into the BSK-H complete medium whereas the longest generation time (about 12 hours) was found in the samples of BSK-H medium. The samples of the BSK-II medium showed a generation time of 11.5 hours during their incubation. Highest growth rate R (0.11h⁻¹) was observed in the samples of BSK-H

complete medium, while the other two media's samples showed lower growth rates R (0.09 h^{-1}) as compared to BSK-H complete medium (Table 1). The samples inoculated in BSK-H medium showed a Lag phase of 35 hours whereas the samples of the other two formulations exhibited a lag phase of 42 hours. The maximum cell density was measured up to 10^9 cells/ml in BSK-H complete medium.

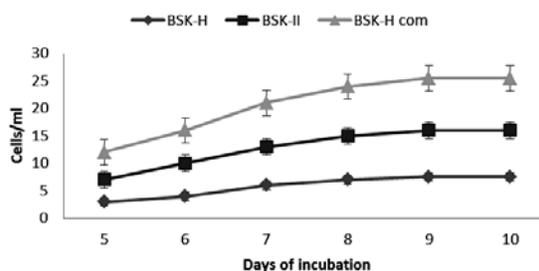


Fig. 3: Growth curve of *B. anserina* in different formulations of BSK medium

Table 1: Growth parameters of *B. anserina* in different formulations of BSK medium

Parameters	Medium Used		
	BSK-H	BSK-II	BSK-H complete
G (h)*	12.00	11.30	8.00
R (h^{-1})**	0.090	0.091	0.111
μ (h^{-1}), specific growth rate	0.072	0.075	0.081

*G (h) = Generation time; **R (h^{-1}) = Growth per hour

DISCUSSION

The present study was conducted to observe the effect of different formulations of BSK medium on the growth of *Borrelia*. Different *Borrelia* grow differently in BSK medium due to their fastidious nature and to some other changes, which an organism has to face while adapting to the artificial supplementation of biological medium (Rodríguez *et al.*, 2007). *Borrelia* has a very complex structure and microbial physiology as well. BSK-H complete medium is supplemented with 6% rabbit serum which is very essential for *Borrelia* to grow because it lacks specific genes responsible for the synthesis of different biochemical substances, which are essential to survive, such as amino acids, fatty acids, enzyme cofactors, and nucleotides. Due to its deficiency in some biosynthetic pathways, *Borrelia* needs serum-supplementation during its *in-vitro* cultivation (Fraser *et al.*, 1997). Another reason for the use of serum supplementation for the growth of *Borrelia* is that it also lacks the ability to elongate long-chain fatty acids. BSK-H complete medium is also supplemented with Sodium bicarbonate as well; it neutralizes the acids produced by the bacteria during its growth, which is very crucial for the maintenance of the medium's pH.

Among different ingredients of BSK medium *N*-acetyl-glucosamine (NAG) is a vital component (Pollack *et al.*, 1993) for the growth of *Borrelia* because it integrates into the cell wall, and serves as an energy source. NAG is the basic element of chitin; it makes the cuticle of the tick vector and act as a source of carbohydrate for *Borrelia*.

In the current study it was observed that BSK-H complete medium has a significant impact on the growth of *Borrelia*. These results are consistent with some previous studies conducted on the growth analysis of *Borrelia*, which showed that there are significant differences among the growth kinetics of *Borrelia* when it is cultivated in different formulations of BSK medium (Wang *et al.*, 2004). The logarithmic phase for *B. anserina* while using the BSK-H complete medium was faster as compared to the other two formulations; the result of this part of the study is in accordance with the results of Rodríguez *et al.* (2007). They found that BSK-H medium has a good effect on the growth as compared to modified Kelly medium, while in this study we used BSK-H complete medium to even further facilitate the growth of the *Borrelia*. The number of cells/ml is also lower in BSK-H and BSK-II medium as compared to the BSK-H complete medium; this again verifies the results of the Rodríguez *et al.* (2007) which showed that the cellular concentration was higher in BSK-H medium. Generation time was observed to be lower in case of BSK-H complete medium (up to 8 hours) whereas in case of BSK-H and BSK-II the generation time was calculated to be 12 and 11.5 hours, respectively. It has been established that depending upon the culture conditions and temperature, the generation time of *Borrelia* ranges from 7 to 20 hours (Preac-Mursic and Wilske, 1993).

Due to the obvious reason of motility the observation of the spirochetes under phase contrast microscope was found very difficult to combat this problem the culture was kept at 4°C , which slows-down the movement of the *Borrelia*. Afterwards, the confirmation of the isolates as *B. anserina* was done by indirect immunofluorescence, a method used in the early detection of *B. burgdorferi*.

Flagellin gene-based PCR has been established to detect *Borrelia* in clinical samples (Lebech and Hansen, 1992). Since it has highly conserved nature among *Borrelia* species the flagellin gene-targeted PCR analysis can be used to detect the infections irrespective of the causative species. According to these findings we also identified all the isolates as *B. anserina* by flagellin gene based PCR.

Our results showed the impact of BSK medium on the cultivation of *Borrelia*. BSK-H complete medium supplemented with 6% rabbit serum and Sodium bicarbonate augmented the growth, reduced the generation time and enhanced growth rate of *Borrelia* spirochaetes as compared to two other formulations of BSK medium.

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