**Babesia bovis**: Effect of Albumax II and orotic acid in a low-serum in vitro culture

Claudia Sánchez¹,*¹, Elisa Campos¹, Abel G. Oliva

Instituto de Biología Experimental e Tecnológica/Instituto de Tecnología Química e Biológica, Universidade Nova de Lisboa, Biomolecular Diagnostics Laboratory, Av. da República (EAN), Apt. 127, 2781-901 Oeiras, Portugal

**1. Introduction**

*Babesia bovis* is a haemoprotozoan parasite of the genus *Babesia* (order Piroplasmida, phylum Apicomplexa) that grows asexually within bovine RBC² (Okamura et al., 2007). Together with *Babesia bigemina*, *B. bovis* appears as responsible for bovine babesiosis, an economically important tick-borne disease of cattle in tropical and subtropical regions of the world. However, it is generally accepted that *B. bovis* is the more virulent of the two organisms (Vial and Gorenflo, 2006). *Babesia divergens* is also another important causal agent of babesiosis in cattle, especially in European countries (Zintl et al., 2003). The clinical signs of this disease vary from inapparent infection in many animals to high fever, severe anaemia and hypotensive shock syndrome leading to multiple organ failure and, even in some cases, death (Palmer, 2002).

The development of a convenient in vitro system for the cultivation of *B. bovis* and the improvement of the MASp culture have facilitated studies on diagnosis, immunity and chemotherapy in babesiosis (Goff and Yunker, 1988).

It is well-known that serum is an essential factor for the in vitro culture of haemoparasites. The critical components may include serum proteins, lipids, sugars, vitamins, growth factors and hormones that synthetic media fail to supply (Grande et al., 1997). However, several attempts have been made to reduce or replace BS in cultures by a combination of more defined components that may offer clear advantages, such as a higher potential for standardization, the simplification of the isolation and purification of target molecules from cultures and, besides, the reduction of risks associated to contaminations by the manipulation of fresh serum (Neves et al., 2001).

Florin-Christensen et al. (2000) found that *B. bovis* infected erythrocytes contain significantly higher amounts of lipids than uninfected erythrocytes cultured under the same conditions. This increase may be explained by the large increment of total membranes associated to the growth of the parasite within the host RBC, which represents a considerable requirement of new membranes associated to the growth of the parasite within the host RBC, which represents a considerable requirement of new membranes (Grande et al., 2007; Zweygarth et al., 1999; Neves et al., 2001).

In vitro studies of the metabolism of nucleic acid precursors by *Babesia* spp. showed that the parasites obtain preformed purines such as Albumax™, an alternative to bovine serum for continuous in vitro cultivation (Grande et al., 1997; Zweygarth et al., 1999; Jackson et al., 2001; Neves et al., 2001).

In vitro studies of the metabolism of nucleic acid precursors by *Babesia* spp. showed that the parasites obtain preformed purines from the host by salvage pathways and pyrimidine by *de novo* pathways. Particularly, *de novo* synthesis of pyrimidines via orotic acid seems to be important in bovine parasites (Irvin and Young, 1979). Gero et al. (1983) reported the activity of the six enzymes involved in the pyrimidine *de novo* biosynthetic pathway in two bovine *Babesia* spp., *B. bovis* and *B. bigemina*.

In the present study, the effect of reducing the BS content in the culture medium on *B. bovis* Mo7 growth was evaluated. Additionally, the supplementation of low-serum media with Albumax™ II
and/or orotic acid and their effect on parasite in vitro growth was also investigated. Furthermore, a study of the morphological characteristics of the parasites cultured in the different media was also described.

2. Materials and methods

2.1. Parasites

The Mo7 biological clone of B. bovis, derived by limiting dilution of the Mexico strain as described elsewhere (Rodriguez et al., 1983; Hines et al., 1989), was kindly provided by Dr. Erik de Vries, from the Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands. Stocks of B. bovis Mo7 cultures were cryopreserved and kept in liquid nitrogen.

2.2. Erythrocytes

Blood from an uninfected adult bovine was collected into sterile tubes (S-Monovette®, Sarstedt) containing sodium citrate as anticoagulant. The RBC were washed in VyMs (Vega et al. 1985) and centrifuged (480g, 15 min, 4 °C) three times. After the first wash, the white blood cells were removed from the interphase of the plasma and RBC suspension. After the third wash, the RBC were suspended in VyMs in a volume proportion of 1:1 and stored at 4 °C for maximally 2 weeks. The VyMs was changed every two days.

2.3. Culture media

The basic culture medium was prepared with M199 with Earle's salts (Gibco®) supplemented with 50 µg/ml gentamycin (Gibco®) and 20 mM TES (Sigma–Aldrich®). The media used for the study of the influence of BS concentration on the growth of B. bovis parasites were constituted by basic culture medium and 0%, 10%, 20%, 30%, 40% and 50% (v/v) of BS. Supplemented media were made by adding 2, 5 and 10 mg/ml of Albumax™ II (Gibco®) to 0%, 10%, 20% and 30% (v/v) serum media. For the experiments performed with orotic acid (Sigma–Aldrich®), concentrations of 10, 25 and 50 µM were used whenever indicated.

BS used throughout this work was obtained by centrifugation of defibrinated blood (480g, 15 min, 4 °C) from an uninfected adult bovine, sterilized by filtration and kept at −20 °C, until use.

2.4. Initiation of B. bovis Mo7 culture and maintenance

The B. bovis culture technique used throughout this work was the microaerophilous stationary phase previously described by Levy and Ristic, (1980). Stocks of B. bovis cryopreserved from previous MASP cultures grown in medium containing 40% of BS were used to initiate new MASP cultures.

Vials of B. bovis stablilates were thawed, in a water bath at 37 °C, and the content was diluted in VyMs. After centrifugation at 480g for 10 min at 4 °C, the pellet was resuspended in basic culture medium with 40% of BS containing fresh uRBC in order to obtain a 5% haematocrit suspension and distributed in a 96-well flat-bottomed microculture plate (Greiner Bio-One). The volume of culture per well was 200 µl in order to guarantee a culture depth of 0.62 cm (Levy and Ristic, 1980). The plate was incubated at 37 °C in a humidified atmosphere of 2% O2, 5% CO2, and 93% N2. Daily, about 60% of the overlying medium was removed without disturbing the layer of settled RBC and replaced by fresh medium (40% of BS). After 48 h, subcultures were made by resuspending the RBC in the culture wells followed by transferring 100 µl (1:2) into each new well. Then, a 5% haematocrit suspension of uRBC in fresh medium was added to make up a final volume of 200 µl. The day after, the plate was transferred to an incubator containing 5% CO2 in air.

2.5. Effect of medium composition on the growth of B. bovis Mo7

MASP cultures already adapted to basic culture medium with 40% of BS were transferred to a 96-well microplate and a certain volume of an uRBC suspension in the several studied media was added. The total volume of culture in each well was 200 µl and the haematocrit was 10%. Every condition (medium composition) was tested in sextuplicate. After 72 h, subcultures were prepared by dilution with a 10% haematocrit uRBC suspension in the respective medium, to obtain a PPE around 1% (Levy and Ristic, 1980). Cultures were maintained during nine days at 37 °C in a humidified atmosphere with 5% CO2 in air.

2.6. Assessment of parasitaemia and study of morphologic characteristics by light microscopy

Daily, two blood smears of each tested condition were prepared. Thin films of blood were air-dried, fixed in absolute methanol, stained in freshly prepared 2% (v/v) buffered Giemsa's stain pH 7.2 for 60 min and, at last, rinsed thoroughly in water and air-dried. Stained smears were viewed by using a Leica® DMRB microscope at 100× magnification under oil immersion. The PPE was determined by counting the number of iRBC in five microscope fields of approximately 800 RBC each and expressed as number of iRBC/100 RBC. For values presented as relative PPE, the following expression was used: Relative PPE = PPEi /100 PPEmax, where PPEi is the average PPE of the corresponding day of the cycle and the PPEmax is the maximum average PPE obtained during the culture.

For morphologic study of B. bovis intraerythrocytic forms, an image analysis setup composed by a Nikon® TE2000-S microscope coupled to an Evolution™ MP Color video camera (MediaCybernetics, USA) was used. Stained smears were viewed at 100× magnification under oil immersion. Digital images were captured through an image analysis software (Image-Pro® Plus 5.1).

2.7. Statistical analysis

Data are expressed as the mean PPE ± standard error (SE). The Student’s unpaired t-test was used to determine the statistical significance of differences between mean values. Significance was defined as P < 0.05.

3. Results

3.1. Effect of BS concentration on B. bovis growth

B. bovis cultures adapted to basic culture medium with 40% of BS were grown in media with 0%, 10%, 20%, 30%, 40% and 50% of BS, at 37 °C in a humidified atmosphere with 5% CO2 in air. The PPE of each condition was followed during three cycles. Each cycle corresponds to three days of culture finishing with the culture splitting. In order to evaluate the overall behaviour of the culture during the cycles, the average of each day in the three cycles was determined. Then, these values were compared with the maximum one and expressed as relative PPE (Fig. 1).

PPE increased markedly from the first to the second day in the cultures grown in media with high BS concentration, while for those with 0% and 10% BS no significant differences (P < 0.05) in PPE were detected. Concerning the variation from the second to the third day, a minor decrease was observed, in general. The highest PPE values were obtained in the culture media with 50% and 40% of BS, being both very similar. Lower PPE were at-
tained in the media with 30%, 20% and, even more, with 10% of BS. Medium without serum addition was not capable to support an acceptable culture growth.

In order to evaluate the effect of oxygen concentration on the growth of B. bovis in media with different BS concentrations, a parallel assay was performed in a low oxygen atmosphere (2% O₂). In this case, the overall behaviour during the culture was very similar to that obtained in an atmosphere 5% CO₂ in air, despite the PPE values were much lower and less dependent on the BS concentration (data not shown).

### 3.2. Supplementation of low-serum media with Albumax™ II

Basic culture media containing 0%, 10%, 20% and 30% of BS were supplemented with 5 and 10 mg/ml of Albumax™ II. The cultures were grown at 37 °C in a humidified atmosphere with 5% CO₂ in air. The aim of this experiment was to investigate if the growth of B. bovis in medium supplemented with Albumax™ II was similar to that observed in rich-serum medium. For this purpose the behaviour of the cultures during the cycles was evaluated by comparing the average PPE of each day in the three cycles with the average PPE obtained for a culture grown under optimum conditions (40% of BS) and in medium without Albumax™ II as controls. The PPE of the different growing media observed during three cycles are presented in Fig. 2.

Albumax™ II supplementation, in all the concentrations tested, slightly improved the growth of B. bovis in media with 30% and 20% of BS at the end of each cycle (Fig. 2A and B, respectively). The PPE values were very close or even higher than those corresponding to 40% BS. Although only in 20% BS medium with 2 mg/ml of Albumax™ II the enhancement was significant (P < 0.05) comparing with the control without addition. This Albumax™ II concentration was indeed the optimum in cultures grown with 20% BS (Fig. 2B).

Addition of Albumax™ II to low-serum medium (10% of BS) improved parasite multiplication, which is revealed by higher PPE values at the end of each cycle (Fig. 2C). For all the concentrations of Albumax™ II tested, no significant differences were observed when comparing with 40% of BS. On the other hand, a considerable enhancement was observed regarding with the control 10% BS (significant differences with 95% confidence).

Finally, despite the addition of Albumax™ II to 0% BS medium, a decreased of the average PPE was observed after the first day of each cycle (Fig. 2D). In general, the overall growth of the B. bovis cultures in these media was very low, and no important differences were observed with the control without addition (P < 0.05).

### 3.3. Supplementation of low-serum media with orotic acid

Orotic acid was added in concentrations of 10, 25 and 50 µM to basic culture media containing 10% and 20% of BS and to the same media supplemented with 5 or 2 mg/ml of Albumax™ II, respectively. The parasites were grown at 37 °C in a humidified atmosphere with 5% CO₂ in air. The average PPE of each day in the three cycles was determined.

Addition of orotic acid to 10% or 20% BS media did not benefit the growth of B. bovis parasites (data not shown). In general, no significant enhancement of PPE was observed (P < 0.05). However, in medium with 20% BS the presence of orotic acid seemed to have a positive effect at the end of the cycles (third day). Particularly, for a concentration of 50 µM of orotic acid, an increment of 25% (P < 0.05) in the average PPE was observed regarding the control without addition (data not shown).

The positive effect previously observed for Albumax™ II supplementation was no evident in presence of orotic acid, since no significant differences (P < 0.05) were observed in the tested conditions (data not shown).

### 3.4. Cytological characteristics of the parasitic forms

Giemsa-stained thin smears of B. bovis parasites after six days of cultivation in different culture media were examined by light microscopy. No morphological alteration was identified in parasites grown in low-serum media, 20% and 10% of BS, when compared with those grown in 40% BS medium (data not shown). Moreover, no change was observed on infected RBC. All the parasitic forms of the intraerythrocytic cycle such as trophozoites, merozoites, and the rarely seen but often described maltese-cross form or tetrad, were visualized in these media (data not shown). In contrast, in cultures grown in 0% BS medium only trophozoites were observed.

Babesia bovis parasites cultured in media supplemented with Albumax™ II or orotic acid did not reveal any cytological differences when compared with those grown in 40% BS medium, at least in all the tested conditions (data not shown).

### 4. Discussion

Several Babesia species have been successfully cultured in vitro. For the cultivation of B. bovis, in particular, Levy and Ristic (1980) developed the MASP technique, using a culture medium with 40% of BS. In the present work, B. bovis Mo7 was continuously cultured in media with different BS concentrations. The growth of the parasites in basic culture medium with 0%, 10%, 20%, 30%, 40% and 50% of BS showed a similar profile of PPE variation during the course of the culture. In general, PPE increased from the first to the second day of each cycle, and diminished from this day to the third (Fig. 1). Earlier works mentioned the same variation. Goff and Yunker (1988) observed that, even with daily medium changes in order to reduce the accumulation of inhibitory metabolites, parasite replication did not prosecute after the PPE reached a threshold level. On the other hand, it is known that the growth of B. bovis in vitro is influenced by the pH of the culture medium. These parasites grow better at slightly alkaline pHs (pH 7.0 to 7.8) than at acidic pH (pH 6.8), with optimal growth occurring within the range of 7.3–7.4 (Goff and Yunker, 1988). In Plasmodium falciparum cul-
The PPE denotes an inverse relationship with the pH; that is, the increase in the PPE provokes a pH shift to acidic conditions due to lactic acid accumulation. Then, this acidification is responsible for the diminution of the PPE in the subsequent days of the culture (Druilhe et al., 1980; Fairlamb et al., 1985). This behaviour observed in *P. falciparum* could also be an explanation for the decrease on the PPE observed in the *B. bovis* cultures.

In general, lower PPE values were obtained when lower BS concentrations were used in the culture medium (Fig. 1). Nevertheless, it is important to note that medium without BS failed to support continuous growth of *B. bovis* parasites. Albeit the parasite could use some nutrients directly obtained from the host RBC (cytoplasm or membranes), the low PPE observed suggests that the parasite needs further serum factors for optimal growth. These results are in agreement with those reported in the past where attempts of serum-free cultures were unsuccessful either for *B. bovis* or for *B. bigemina* (Neves et al., 2001). Even so, various Babesia spp., like *B. caballi* (Zweygarth et al., 1999), *B. divergens* (Schrével et al., 1992; Grande et al., 1997), *B. equi* (Zweygarth et al., 1996) and *B. occultans* (Zweygarth et al., 1995), have been already grown in vitro in serum-free medium.

Important mechanisms such as RBC invasion and parasite maturation and multiplication, imply an increase on lipids demand (Grande et al., 1997; Vial et al., 2003). Normally, these lipids requirements are satisfied by the serum added to the culture medium. Since parasitaemia in low-serum media cultures was inferior to the one routinely observed with 40% of BS medium, we supplemented these media with Albumax™ II in order to restore the PPE. In general, the positive effect of Albumax™ II supplementation was only evidenced at the end of the cycles. During the first two days of each cycle, BS concentrations of 30% and 20% seemed to supply all the nutrients needed to support *B. bovis* growth, since no significant differences with the controls (no addition) were observed.

The benefit of Albumax™ II was clearly observed in medium with 10% of BS. The low-serum concentration present in this medium was unable to support a satisfactory parasite growth; however, the Albumax™ II addition was fundamental to restore the PPE at the end of the cycles. In despite of the PPE obtained were analogous to that attained with 40% of BS medium, the difference observed suggests that serum would afford other still-undetermined components needed for the optimal growth of the parasite.

Aiming to further enhance the growth of *B. bovis* in low-serum media, new attempts were made by adding orotic acid to the medium. In general, we observed that orotic acid added to the 10% or 20% of BS culture media, alone or in combination with Albumax™ II, did not improve notably the PPE. Nevertheless, in 20% BS media, the addition of orotic acid seemed to have a beneficial effect at the end of the cycles. These data suggest that orotic acid would promote parasites growth in media composed by moderate BS concentrations and later in the culture, which is advantageous when performing a continuous culture.

Finally, the different stages of the intraerythrocytic cycle of *B. bovis* were identified in all the tested media, which means that the parasite underwent the typical development phases in all the conditions. Exceptionally, in cultures maintained with serum-free medium, only trophozoites were viewed, which jointly with the lower PPE values observed in this condition, confirm the unfeasibility of achieving a suitable *B. bovis* growth in this medium.
In this paper, we showed that higher PPE values were obtained for media with 40% and 50% of BS. The poor B. bovis growth reached with low-serum media (10% BS) was improved by the addition of Albumax™ II. On the other hand, the combination of Albumax™ II and orotic acid at the concentrations used was not a good alternative to substitute BS. Given that parasitaemia was not restored to the levels comparable to those obtained with 40% of BS media, other BS critical components are surely involved in the optimal growth of B. bovis. Further studies must be carried out in order to better understand the role of serum components and to explore the development of a reduced or serum-free chemically defined culture medium for B. bovis.

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