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Babesia bigemina: Advances in continuous *in vitro* culture using serum-free medium supplemented with insulin, transferrin, selenite, and putrescine

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ABSTRACT

This study reported that *Babesia bigemina* (Bbig-SF) was continuously cultured *in vitro* in a serum-free medium supplemented with a mixture of insulin-transferrin-selenite (M-ITS) and putrescine (Pu). Firstly, the effect of five different types of basal culture media supplemented with 40% bovine serum was evaluated regarding the proliferation of the protozoan parasite. Cultures with the advanced DMEM/F12 medium (A-DMEM/F12) showed the highest percentage of parasitized erythrocytes (PPE) at 8.37%. Using A-DMEM/F12, a strain of *B. bigemina* (Bbig-SF) was adapted for growth in bovine serum-free medium by a sequential reduction of serum and demonstrated a maximum PPE of 7.18% in the *absence* of serum. The next study was the evaluation of the effect of adding four different concentrations of M-ITS to the serum-free A-DMEM/F12 medium on Bbig-SF; the optimal concentrations of M-ITS were 2000, 1100, and 1.34 mg/L, which yielded a PPE of 7.23%. Next, eight levels of Pu were evaluated on Bbig-SF cultured in serum-free A-DMEM/F12. After the addition of 0.1012 mg/L of Pu, the maximum PPE was 7.61%. When the combination of serum-free A-DMEM/F12 + M-ITS (2000, 1100, and 1.34 mg/L) + Pu (0.1012 mg/L) was evaluated, it yielded a maximum PPE of 14.80%. Finally, the combination of M-ITS + Pu in A-DMEM/F12 without serum and incorporation of a perfusion bioreactor yielded a maximum PPE of 33.45%. We concluded these culturing innovations for *B. bigemina in vitro* allow the optimization of small- and large-scale proliferation as a source of this protozoan parasite for future studies.

1. Introduction

Bovine babesiosis is principally caused by *Babesia bovis* and *Babesia bigemina*, which are protozoan parasites that invade the erythrocytes of the vertebrate host. This disease is widespread in tropical and subtropical regions of the world and causes great economic hardship due to the losses in milk and meat production. These protozoa are transmitted by *Rhipicephalus* (*Boophilus*) *microplus* and *R. (Boophilus) annulatus* ticks [1,2].

There are no laboratory animal models that allow the proliferation of *B. bigemina*. Thus, to obtain these parasites, it is necessary to use bovine inoculation or *in vitro* culture; the latter was established by Vega et al. [3]. This procedure has allowed researchers to obtain biological material for diagnostic purposes [4], drug evaluation [5], and the production of strains with immunogenic capacity [6]. Few changes have been made to the original method used to culture this protozoan, which was developed 30 years ago. The standard procedure uses the M-199 culture medium with Earle's salts supplemented with a high level of adult bovine serum (20–40% v/v) [7]. Serum is required for the proliferation of the parasite; however, its composition presents very significant qualitative and quantitative variations between batches [8,9,10]. Furthermore, it contains toxic compounds such as proteases, free radicals, and growth inhibitors that affect the proliferation of these parasites [11,12].

So far, there have been very few studies that attempt to reduce or eliminate the use of serum in the *in vitro* culture of *B. bigemina*, which is an extremely demanding and difficult technique [13,14].Thus, it would be very useful to find new methods that replace serum with essential elements that enable the optimal growth of *B. bigemina*.

Recently, Rojas et al. [15] demonstrated that by using the advanced

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DMEM/F12 (A-DMEM/F12) medium supplemented with a mixture of insulin-transferrin-selenite (M-ITS) it is possible to support the increased proliferation of *B. bovis* in a culture medium free of bovine serum; however, it has not been possible to completely eliminate serum from the culture medium in the case of *B. bigemina* [3,13,14].

Moreover, it has been reported that polyamines such as putrescine, spermine, and spermidine are aliphatic molecules involved in the proliferation of protozoan cells and parasites [16]. For example, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania* spp., and *Plasmodium* spp. depend on the presence of specific amounts of polyamines for proliferation [17–19]. Very recently, Rojas-Martínez et al. [20] demonstrated that the A-DMEM/F12 medium without bovine serum and supplemented with putrescine (Pu) support the *in vitro* proliferation of *B. bovis*. Regarding *B. bigemina*, there are no reports on the effect of polyamines on its proliferation. Thus, the specific aims of this work were to find an alternative to the standard basal medium, to eliminate bovine serum from the culture medium, and to determine the optimal concentration of the M-ITS and Pu separately and the combination of M-ITS + Pu in A-DMEM/F12 medium for the proliferation *in vitro B. bigemina*. The final objective was to evaluate the large-scale use of the new formulation in a hollow-fiber perfusion bioreactor.

2. Materials and methods

2.1. Basal culture media and supplements

Five commercial culture media were used: advanced DMEM (A-DMEM), advanced MEM (A-MEM), advanced RPMI 1640 (A-RPMI), advanced DMEM/F12 (A-DMEM/F12), and M-199 with Earle's salts (M-199) (GIBCO® Grand Island, NY, USA). The supplements used were 40% (v/v) adult bovine serum, 25 mM TES 2-[(2-hydroxy-1, 1-bis (hydroxymethyl) ethyl) amino] ethanesulfonic, *N*-[Tris (hydroxymethyl) methyl]-2-aminoethanosulphonic (Sigma-Aldrich, St. Louis, MO, USA), and 2 mM L-glutamine (GIBCO®). Four concentrations of the insulin, transferrin, and selenite (M-ITS) (GIBCO®) mixture were used (Table 1). Eight concentrations of putrescine (Pu) were also evaluated (0.0063–0.810 mg/L) (Sigma-Aldrich). The cryoprotectant used was polyvinylpyrrolidone-40 (PVP-40) (Sigma-Aldrich) at 20% (w/v) in A-DMEM/F12 medium. The solution used for washing and storage of erythrocytes was A-DMEM/F12 + antioxidant mixture (Sigma-Aldrich). The pH of the media and supplements was adjusted to 6.8 with 1 M HCl. They were sterilized by filtration with a 0.22 μm membrane (Millipore).

2.2. Experimental animals

Erythrocytes and serum were obtained from two one year-old *Bos taurus* (Holstein Friesian) bovines free of *Anaplasma marginale* and *Babesia* spp. To confirm the absence of *Babesia bovis*, *B. bigemina,* and *A. marginale*, bovine blood samples were analyzed utilizing the nested polymerase chain reaction (n-PCR) as reported by Figueroa et al. [21]. The animals were handled according to the guidelines for good management and animal welfare established by NOM-062-ZOO-1999 (Technical specifications for production, care, and use of laboratory animals) in México (http://www.senasica.gob.mx/?doc=743).

Table 1
Addition of mixtures of insulin-transferrin-selenite to the basal A-DMEM/F12 medium for the *in vitro* proliferation of *Babesia bigemina*.

A-DMEM/F12	I	II	III	IV	M-199[a]
Mixtures					0
I	500	1000	2000	4000	0
T	250	550	1100	2200	0
S	0.335	0.67	1.34	2.68	0

I insulin; T transferrin; S selenite; each component concentration is expressed as mg/L.
[a] As a control M-199 culture medium supplemented with 40% (v/v) bovine serum.

2.3. Erythrocytes and serum

The blood was collected from the jugular vein and defibrinated with glass beads. Serum separation was performed by centrifugation at 450 × *g* at 4 °C for 30 min. The serum was stored at − 20 °C. The buffy coat was discarded and the collected erythrocytes were washed three times with A-DMEM/F12 + antioxidant. They were then resuspended in the same solution in a 1:2 proportion and stored at 4 °C. Thereafter, the erythrocytes and serum were irradiated with a cobalt-60 source at 70 Gy and 25 kGy, respectively [22]. Irradiated erythrocytes were stored at 4 °C and irradiated serum was aliquoted into 20 ml aliquots and stored at − 20 °C until use.

2.4. In vitro culture of Babesia bigemina

One cryostabilate of an attenuated strain of *B. bigemina* (BIS) [22] containing 1 × 10^8 infected erythrocytes in 1 ml was removed from the liquid nitrogen, thawed at 30 °C, and resuspended in 50 ml of medium M-199 + 40% bovine serum. Afterwards, the erythrocytes were centrifuged at 450 × *g* and 30 °C for 30 min. The supernatant was discarded, and the resulting pellet was resuspended in 800 μl of erythrocytes (10% v/v) in medium M-199 + 40% (v/v) bovine serum and transferred to a 24-well plate. The culture was maintained in an incubator at 37 °C with a gas mixture of 90% N_2, 5% CO_2, and 5% O_2 at constant pressure. The various assays were initiated when the *in vitro* culture of *B. bigemina* was in log-phase proliferation with a percentage of parasitized erythrocytes (PPE) over 5%. Routine management consisted of replacing the culture medium every 24 h, subculturing when any treatment reached a PPE of 4%, and adjusting to 1% to reinitiate cultures. When the PPE was equal to or lower than 1%, a 1:2 subculture dilution with the 10% donor erythrocytes was performed. Thin smears fixed with absolute ethanol and stained with Giemsa were prepared from each well to monitor the proliferation of parasites. The PPE was calculated by counting 5000 erythrocytes per smear. The experiments were conducted in triplicate with three replicates.

2.5. Selecting an optimal culture medium for the in vitro proliferation of Babesia bigemina

Five basal culture media were evaluated: A-DMEM, A-MEM, A-RPMI, A-DMEM/F12, and M-199. The media were supplemented with 40% (v/v) bovine serum. The initial PPE was 1%. The culture supernatant was changed every 24 h, and the subcultures were carried out when the PPE reached 4% with an adjustment to 1% to reinitiate cultures.

2.6. Elimination of bovine serum from the in vitro culture of Babesia bigemina

The basal medium used was A-DMEM/F12, and M-199 was used as a control. The concentration of serum was gradually reduced: 40, 30, 20, 10, 5, 1, 0.5, and 0% (v/v) for both media. The criteria for reducing the concentration of serum was cultures with a PPE of 4% or higher over a minimum of three consecutive subcultures. For each concentration of bovine serum utilized, the parasites were adapted. Infected and uninfected red blood cells were washed twice with A-DMEM/F12 before proceeding to the next concentration of serum. *B. bigemina* that adapted to and proliferated in the serum-free medium were renamed Bbig-SF.

2.7. Effect of adding the mixture of insulin, transferrin, and selenite (M-ITS) to the A-DMEM/F12 medium on the in vitro proliferation of Babesia bigemina

In this assay, Bbig-SF was cultured in A-DMEM/F12 supplemented with four concentrations of M-ITS (Table 1). The control treatment was *B. bigemina* (BIS) cultured in medium M-199 supplemented with 40% (v/v) bovine serum.

2.8. Effect of adding different concentrations of putrescine (Pu) to the serum-free A-DMEM/F12 on the in vitro proliferation of Babesia bigemina

Bbig-SF was cultured *in vitro* in serum-free A-DMEM/F12 supplemented with eight different concentrations of Pu: 0.006, 0.013, 0.025, 0.051, 0.101, 0.203, 0.405, and 0.810 mg/L. The control treatment was *B. bigemina* (BIS) cultured in medium M-199 supplemented with 40% (v/v) bovine serum.

2.9. Effect of the combination of Pu and M-ITS to the serum-free A-DMEM/F12 on the in vitro proliferation of Babesia bigemina

Bbig-SF were used, and the A-DMEM/F12 medium was supplemented with M-ITS (insulin 2000 mg/L; transferrin 1100 mg/L; selenite 1.34 mg/L) and Pu (0.101 mg/L). The control treatment was *B. bigemina* (BIS) cultured in medium M-199 supplemented with 40% (v/v) bovine serum. The culture was carried out as previously described by Rojas-Martínez et al. [20].

2.10. Large-scale in vitro proliferation of Babesia bigemina in a hollow-fiber perfusion bioreactor system (HFPBS)

Bbig-SF was used for this assay, and the cultured medium was A-DMEM/F12 + Pu + M-ITS. Erythrocytes (1 ml) with a PPE of 4% were transferred to a 75 cm^2 culture flask, and 50 ml of culture medium with 10% (v/v) uninfected erythrocytes were then added. When a PPE of 7% was reached, the contents of the flask were centrifuged at 450 × *g* and 30 °C for 30 min. The resulting cell pellet (5 ml) was mixed with 45 ml (v/v) of uninfected erythrocytes and was then resuspended in 50 ml of serum-free A-DMEM/F12 + Pu + M-ITS. This final suspension was transferred to the HFPBS (FiberCell® System, MD, USA).Two hundred and fifty milliliters of A-DMEM/F12 + Pu + M-ITS were in constant circulation [23]; the HFPBS was maintained in an incubator at 37 °C with a gas mixture of 90% N_2, 5% CO_2, and 5% O_2 at constant pressure. The erythrocytes contained in the cartridge were removed and washed with A-DMEM/F12 medium. The culture medium was replaced every 24 h (250 ml) [20]. Similar culture conditions were followed for the control treatment, where medium M-199 + 40% bovine serum was. Infected erythrocytes were harvested every 24 h. After every harvesting, cultures were restarted by using 5 ml of erythrocytes 7% infected and 45 ml (v/v) of uninfected erythrocytes as previously described. The PPE was monitored daily by microscopic examination of thin smears stained with Giemsa.

2.11. Cryopreservation of Babesia bigemina adapted to proliferate in serum-free medium

Erythrocytes were extracted from the HFPBS and centrifuged at 450 × *g* and 30 °C for 30 min. The supernatant was then discarded and the pellet was cryopreserved. Briefly, the erythrocytes were washed twice with A-DMEM/F12 medium, and the sediment cells were resuspended in one-fifth of the volume of the cryopreservation solution, which consisted of A-DMEM/F12 medium containing 20% (w/v) PVP-40 + 40% (v/v) bovine serum that constantly agitated to mix the contents. This suspension was left undisturbed for 10 min at room temperature for equilibration before adding the rest of the cryopreservation solution for a final proportion of 1:2 (v/v). The suspension was distributed in 2 ml cryogenic vials and kept at − 70 °C for 24 h and then transferred to liquid nitrogen. Cultured *B. bigemina* in control medium were washed with medium M-199 and cryopreserved as indicated, but M-199 + 20% (w/v) PVP-40 + 40% (v/v) bovine serum was used to store the harvested material.

2.12. Statistical analysis

The statistical analysis consisted of a comparison between the mean values of PPE. This was done by analysis of variance (ANOVA) with a significance level of $P < 0.05$. A Student's *t*-test was used to assess the effect of A-DMEM/F12 medium with supplements compared to medium M-199, with a significance level of $P < 0.05$.

3. Results

3.1. Selecting an optimal basal medium for the proliferation in vitro of B. bigemina

Of the five evaluated media, parasites cultured in A-DMEM/F12 showed the best PPE when compared to maximum growth of parasites in the control medium M-199 (8.37% *vs.* 2.38%). Parasites grown in the other media tested had maximum PPE values as follows: A-DMEM (2.85%), A-MEM (4.22%), and A-RPMI (4.86%). At 7 days post-initiation of culture, only the A-DMEM/F12 medium was able to maintain a constant increase of the *B. bigemina* proliferation with subcultures performed every 24 h (Fig.1).

3.2. Elimination of bovine serum in the in vitro culture of B. bigemina

B. bigemina was successfully adapted to proliferate in a serum-free medium. A maximum PPE of 7.09% was reached 4 days post-initiation of culture with the serum-free A-DMEM/F12 medium. Using the control medium (M-199), the concentration of serum could only be reduced to 20% with a PPE of 0.15%. Three days after culture initiation, subcultures were carried out every 24 h for each of the different bovine serum concentrations (Fig. 2). The subcultures were performed when the PPE was greater than or equal to 4% and was adjusted down to 1%. No apparent morphological alterations in parasite morphology were detected under microscopic observation (not shown).

3.3. Effect of the addition of the mixture of insulin, transferrin, and selenite (M-ITS) to the A-DMEM/F12 medium on the proliferation of Babesia bigemina in vitro

The M-ITS III (I 2000, T 1100, S 1.34 mg/L) yielded a maximum PPE of 7.23%, which is a significantly higher result compared to the PPEs determined in cultures with other treatments ($P < 0.05$). Subcultures were carried out every 24 h during the 10 days of the experiment. The maximum PPE values obtained with the other mixtures were: MI (4.6%), M-II (4.83%), and M-IV (3.67%) (Fig. 3). For the control treatment (M-199 with 40% serum), the maximum PPE reached was 4.5% 1 day post-initiation of culture; it then decreased, and no parasites were observed by optical microscopy after day 7 (not shown).

3.4. Effect of the addition of different concentrations of putrescine to the serum-free A-DMEM/F12 on the in vitro proliferation of B. bigemina

The optimal concentration of Pu that support the highest proliferation of Bbig-SF in cultures was 0.101 mg/L, which yielded a maximum PPE of 7.61% at day 10 post-initiation of culture, which was significantly higher than the PPEs determined for the other culture treatments ($P < 0.05$). This significant difference was observed in seven consecutive subcultures over a period of 10 days (Fig. 4). The parasites showed no apparent morphological changes under light microscopy observation (not shown). The control medium (M-199 + 40%

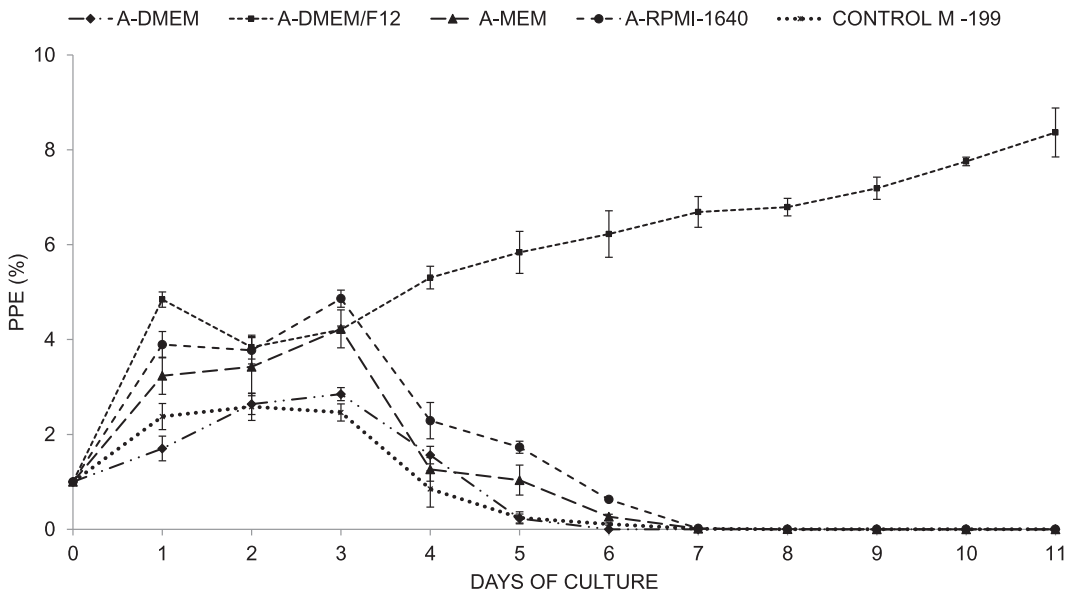

Fig. 1. Evaluation of five culture media supplemented with 40% bovine serum for the *in vitro* proliferation of *B. bigemina*. Each value represents the mean and standard deviation of the procedure in triplicate. Average percentages of parasitized erythrocytes (PPE) obtained with the culture media used in ten subcultures. Subcultures were carried out when the PPE reached 4% and then adjusted to 1%. If the PPE was equal to or lower than 1%, a 1:2 subculture dilution was then performed. Subcultures are not shown in the figure.

bovine serum) yielded a maximum PPE of 3.12% at day 2 post-initiation of culture. No parasites were observed under microscopic analysis after day 6. In cultures with Pu concentrations lower than 0.0506 mg/L, the maximum PPE was 0.33% at 24 h post-initiation of culture. No parasites were observed after 5 days. Morphological changes in anaplasmoid forms were observed in the parasites at concentrations of 0.405 mg/L or higher, and the maximum PPE was 0.38%. No parasites were observed after day 4.

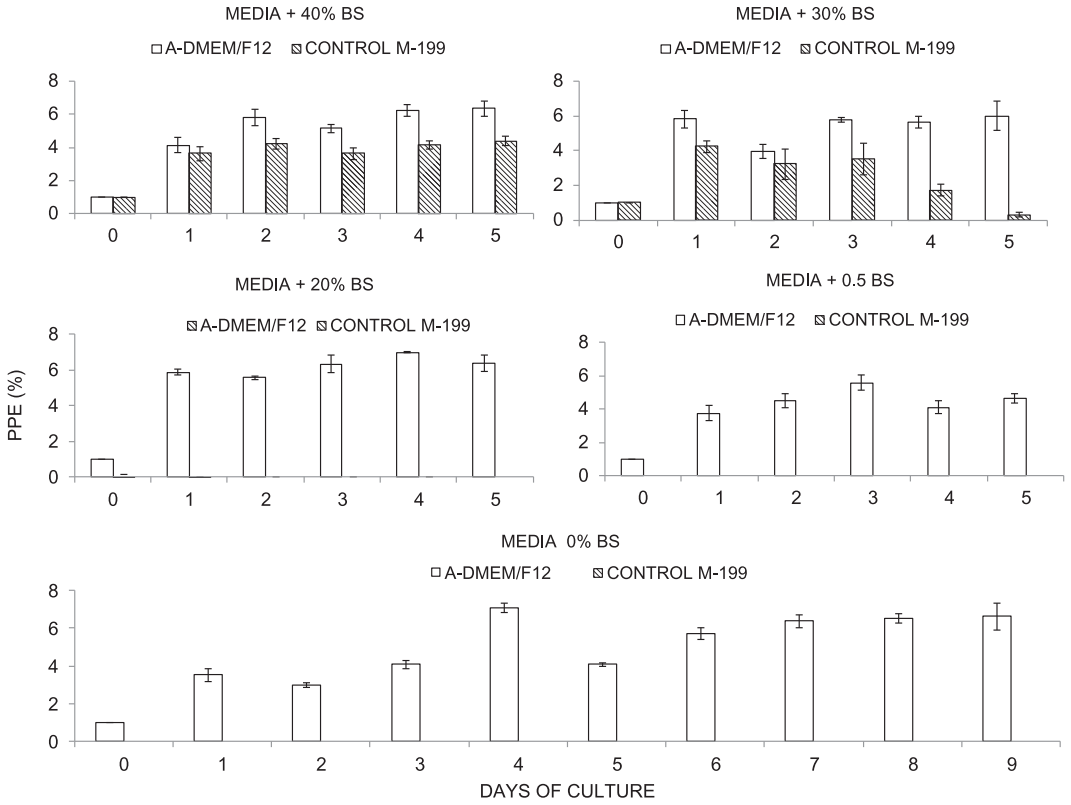

Fig. 2. Sequential adaptation of *B.bigemina* to grow in serum-free A-DMEM/F12 medium. Bovine serum (BS) concentrations used in culture medium: 40%, 30%, 20%, 10%, 5%, 1%, 0.5%, and 0%. These values represent the mean of each treatment and the standard deviation of the procedure in triplicate. Whenever a culture treatment reached a percentage of parasitized erythrocytes (PPE) greater than or equal to 4%, it was adjusted to 1% with 10% of erythrocytes + A-DMEM/F12 (v/v). When the treatments had a PPE lower than or equal to 1%, a dilution was performed (1,2). Subcultures were carried out every 24 h 3 days after starting the use of serum-free medium. The subcultures are not indicated in the graphs. At least five consecutive subcultures were carried out with each higher concentration of BS before using the next lower concentration of BS.

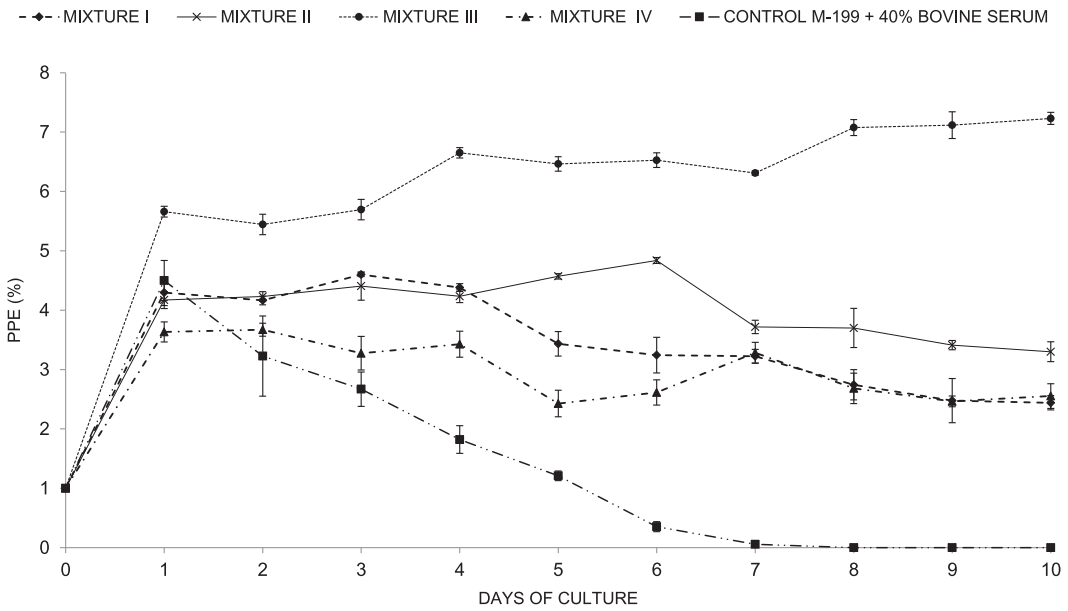

Fig. 3. Evaluation of the addition of four concentrations of the mixture of insulin, transferrin, and selenite (ITS) to serum-free A-DMEM F/12 medium on the *in vitro* proliferation of *B. bigemina*. (Bbig-SF). Mixtures: MI 500-250-0.335; MII 1000-550-0.67; MIII 2000-1100-1.34; MIV 4000-2200-2.68. The control treatment was *B. bigemina* (BIS) in M-199 supplemented with 40% (v/v) of bovine serum (BS). The values represent the mean and standard deviation of each treatment with the procedure in triplicate. Subcultures were made when cultures of any treatment reached a percentage of parasitized erythrocytes (PPE) of 4% and then adjusted to 1% (not shown). When the PPE was lower than or equal to 1%, a 1:2 culture dilution was made.

3.5. Effect of the combination of the mixture of insulin, transferrin, and selenite and putrescine to the serum-free A-DMEM/F12 on the in vitro proliferation of B. bigemina

The continuous *in vitro* proliferation of Bbig-SF was successfully achieved using the combination of A-DMEM/F12 culture medium supplemented with Pu (0.101 mg/L) and M-ITS (2000, 1100, 1.34 mg/L). The maximum PPE yielded in cultures with this treatment was significantly higher ($P < 0.05$) than the control treatment (M199 + 40% bovine serum) (14.80% *vs.* 4.12%). The effect of A-DMEM/F12 + ITS + Pu on parasite cultures allowed the continuous cultivation of Bbig-SF having to perform subcultures every 24 h. It was not necessary to pre-adapt the parasite population to this culture medium, and no morphological changes were observed by light microscopy (Fig. 5).

3.6. Large-scale proliferation of B. bigemina in a hollow-fiber perfusion bioreactor system (HFPBS) using serum-free A-DMEM/F12 + ITS + Pu

The use of an HFPBS and medium with A-DMEM/F12 supplemented with Pu and M-ITS to culture Bbig-SF allowed a maximum PPE yield of 33.45%. This yield was obtained after three subcultures post-initiation of culture with a frequency of subcultures every 24 h. Parasites grown in the HFPBS with the control (M199 + 40% bovine serum) yielded a maximum PPE of 28.75% at day 5. The first subculture of parasites

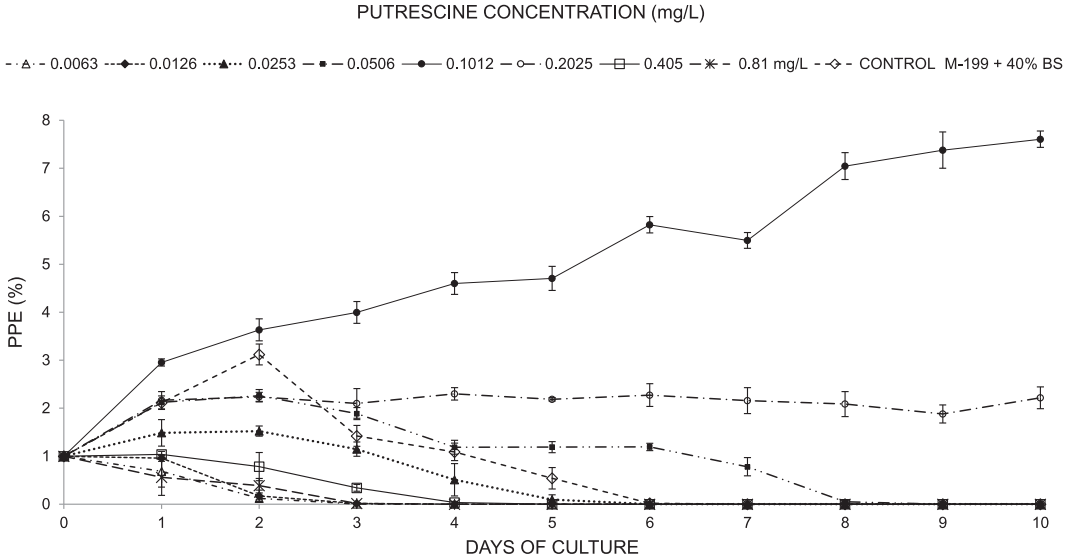

Fig. 4. Evaluation of eight putrescine concentrations in serum-free A-DMEM/F12 for the *in vitro* proliferation of *B. bigemina* (Big-SF). The control treatment was *B. bigemina* (BIS) in medium was M199 + 40% bovine serum (BS). PPE: percentage of parasitized erythrocytes. The values represent the mean standard deviation of the procedure in triplicate. The subcultures are not represented in the graph; they were performed when any treatment reached a PPE of 4% (adjusted to 1%). When the PPE was lower than or equal to 1%, a 1:2 culture dilution was made.

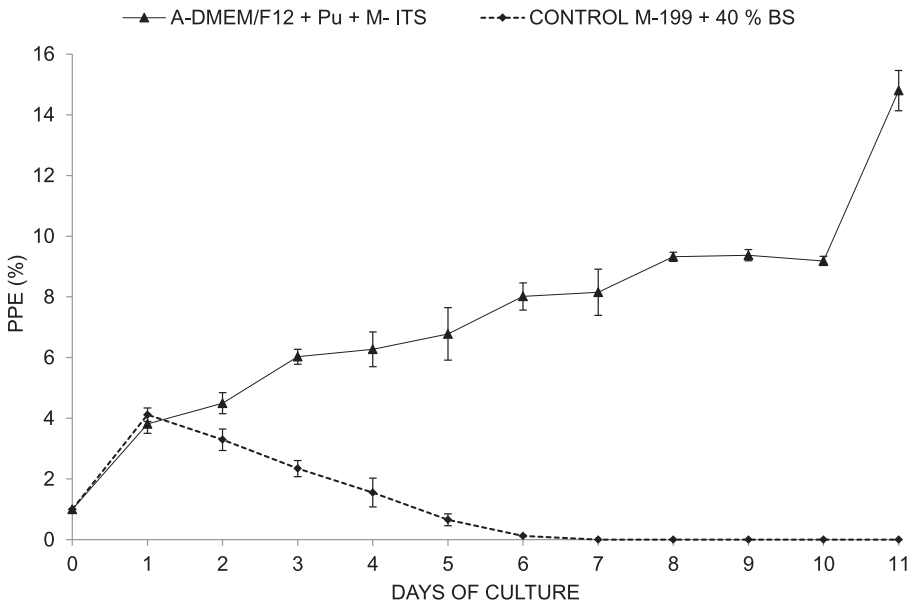

Fig. 5. Evaluation of A-DMEM/F12 serum-free + Pu (0.1012 mg/L) + M-ITS (I 2000, T 1100, S 1.34 mg/L) on the proliferation of *B. bigemina* (Bbig-SF). compared to BIS in M-199 + 40% bovine serum (BS). PPE: percentage of parasitized erythrocytes. The values represent the mean and standard deviation of three repetitions for each treatment. Subcultures were made every 24 h (not shown).

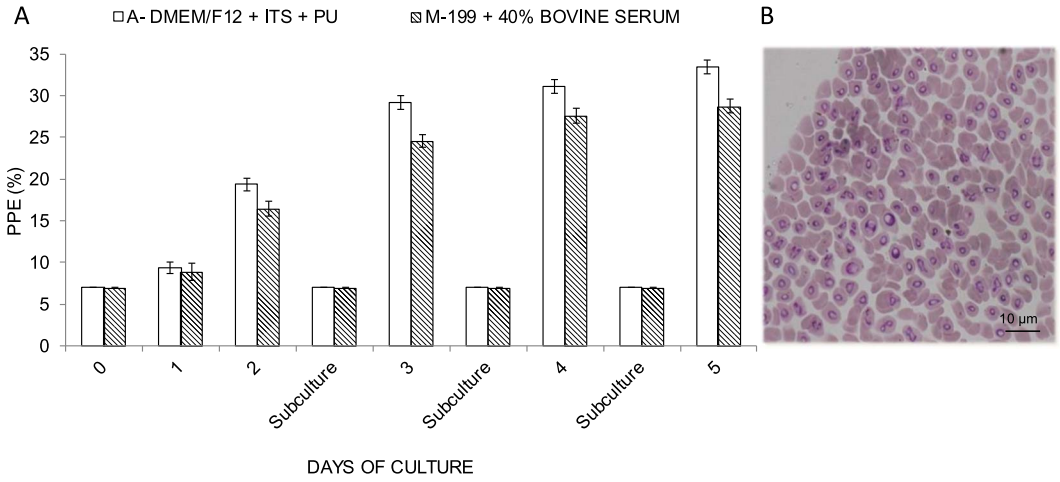

Fig. 6. Large-scale *in vitro* proliferation of *B. bigemina* (Big-SF) in a HFPBS using A-DMEM/F12 medium + putrescine (Pu) (0.1012 mg/L) + insulin, transferrin, and selenite (ITS) (2000, 1100, 1.34 mg/L respectively) without bovine serum (BS). The control treatment was *B. bigemina* (BIS) in medium that was M199 + 40% BS; A) the values represent the mean and standard deviation of three repetitions. PPE: percentage of parasitized erythrocytes. In each subculture, the PPE was adjusted to 7% with a suspension of uninfected erythrocytes of 10% (v/v) in the corresponding culture medium. B) *B. bigemina* harvested from the HFPBS using A-DMEM/F12 + Pu + M-ITS showed no morphological change under optical microscopy.

grown in the HFPBS with the medium A-DMEM/F12 supplemented with M-ITS and Pu was performed at day 2, and then every 24 h thereafter, reinitiating with a PPE of 7% (Fig. 6A). No morphological changes in cultured Bbig-SF parasites with both media were observed by light microscopy (Fig. 6B). The erythrocytes extracted from the HFPBS with A-DMEM/F12 medium were cryopreserved in 2 ml vials, each with a concentration of 1×10^8 infected erythrocytes. Three months after cryopreservation, a cryovial from each frozen batch was thawed and cultures were reinitiated in the same medium that they were cultivated in prior to cryopreservation (data not shown).

4. Discussion

This study demonstrated for the first time that the removal of bovine serum from traditional culture medium for the *in vitro* cultivation of *B. bigemina* (Bbig-SF), can be achieved for a very fastidious *Babesia* species for successful *in vitro* culture proliferation. Furthermore, the use of A-DMEM/F12 supplemented with specific concentrations of the M-ITS and Pu made it possible to greatly increase the proliferation rate of the Bbig-SF parasites in culture plates and obtain an even higher rate in a HFPBS.

The use of an alternative culture medium consisting of A-DMEM/F12 + 40% bovine serum yielded a PPE of 8.37%. The intervals between subcultures were shortened to 24 h 3 days post-initiation of culture. These results differ from those reported in other studies that used the standard culture medium for *Babesia* parasites (M-199 + 40% bovine serum) in which a PPE of 2.5% was obtained, and subcultures were performed every 72 h [3,24]. By using the A-DMEM/F12 medium and sequentially reducing the concentration of serum from 40% down to 0%, it was possible to adapt *B. bigemina* to proliferate in a culture medium free of bovine serum with a maximum PPE of 7.08% at day 4 post-initiation of culture. This study demonstrates for the first time that bovine serum was completely removed from the culture medium for the successful and continuous *in vitro* proliferation of *B. bigemina*. The parasites were likely able to adapt due to the gradual reduction of the bovine serum concentration and because the A-DMEM/F12 medium contains the minimum essential components that can replace bovine serum. The addition of the ITS mixture was also a key factor [25,26]. Moreover, the commercial A-DMEM/F12 medium contains hypoxanthine, a factor derived from adult bovine serum that promotes cell

growth. Hypoxanthine is a purine that some protozoans [27], including *B. bovis*, cannot synthesize *de novo* but require it for proliferation and DNA synthesis [28]. A study by Neves et al. [13] using RPMI medium supplemented with hypoxanthine managed to reduce the concentration of bovine serum down to 10% and successfully cultured *B. bigemina*. Similar results were obtained in an experiment that studied *in vitro* cultures of *Plasmodium falciparum* that demonstrated increased proliferation in a serum-free medium [29,30]. In the cultivation *in vitro* of *B. caballi*, supplementation the HL-1 culture medium with hypoxanthine, lipids, and Albumax I allowed the removal of the equine serum [31]. In the current study, it was not possible to completely remove bovine serum when using the M-199 medium. *B. bigemina* was only able to proliferate in cultures having a serum concentration of 20%. Other studies have reported that this standard medium supports the proliferation of the parasite when it is supplemented with 40% bovine serum and subcultures are performed every 72 h [3].

When the A-DMEM/F12 medium was supplemented with the optimum concentration of the M-ITS (2000 1100, 1.34 mg/L, respectively), it supported the proliferation of the Bbig-SF parasites 24 h post-initiation of culture. The PPE increased constantly until it reached 7.23% on day 10 post-initiation of culture and performing subcultures every 24 h. No apparent morphological changes were observed by light microscopy. It was previously reported that the ITS mixture is essential to reduce or eliminate the concentration of serum in the *in vitro* cultivation of different cell lines; however, the optimal concentration of ITS mixture might be different for each cell and organism [12,25,32]. Rojas et al. [15] reported that by adding the M-ITS to serum-free A-DMEM/F12 for the *in vitro* cultivation of *B. bovis*, it provided a parasite yield PPE of up to 9.7% as compared to the PPE value of 7.23% obtained in this study with *B. bigemina*. This result suggests that, although the conditions were essentially the same, the increase in PPE depends on the species of parasite grown *in vitro*. Previous reports mentioned that by using GIT culture medium that contains ITS, it was possible to stimulate the proliferation of *P. falciparum* without serum [29]. The ITS mixture fulfills specific functions. Insulin facilitates glucose intake by the cells.

It has been shown that erythrocytes infected with *P. falciparum* absorb 26 times more glucose than uninfected erythrocytes [33]. Transferrin is a protein of the globulin group that can capture, collect, and transport iron to different cells and tissues. Iron is important for the synthesis of hemoglobin in erythrocytes. Hemoglobin represents 95% of the cytosolic proteins of erythrocytes, and between 60% and 80% is consumed during the intraerythrocytic cycle of *P. falciparum* [34,35]. Sodium selenite is essential for various important biological functions, such as antioxidant protection by selenoenzymes, specific inhibition of tumor cell growth by metabolites, modulation of the apoptosis cell cycle, and DNA repair [36]. Müller et al. [37] mentioned that the development of *P. falciparum* within a cell requires effective antioxidant systems since an infected erythrocyte is vulnerable to oxidative stress caused by the degradation of hemoglobin [38]. Gamain et al. [39] observed an increase in the number of parasites when supplementing selenium in the culture medium for *P. falciparum* and higher glutathione peroxidase activity when the parasites had undergone oxidative stress. The study showed that this enzyme is dependent on the concentration of selenium. Even though high concentrations of selenium cause cytotoxicity in *P. falciparum* [40], reports indicate that sodium selenite with copper sulfate can be used as a new antimalarial therapy [41].

When the A-DMEM/F12 medium was supplemented with the optimal putrescine concentration (0.1012 mg/L), the PPE reached its highest value at 7.61%. This PPE value was higher than the 6.23% reported for *B. bovis* under the same *in vitro* conditions and at the same concentration. Morphological alterations, such as anaplasmoid forms, were observed in *Babesia* parasites cultured in medium containing Pu concentrations higher than 0.2025 mg/L [20]. Müller et al. [42] showed that the proliferation of *P. falciparum* was dependent on the concentration of polyamines. Previously, Rzepczyk et al. [43] reported high concentrations of spermine (1 mM) and the polyamine oxidase (100 μg/ml) in the *in vitro* culture of *P. falciparum*. Reports also indicate that this enzyme stimulates its proliferation *in vitro* during the intraerythrocytic stage [18,37]. High concentrations of polyamines have also been associated with pathogenicity [44]. *In silico* analysis of the genome sequence indicates that it does not synthesize polyamines *de novo* [28]; thus, *B. bovis* possibly obtains them from the culture medium, the serum, or from bovine erythrocytes. The biosynthesis of polyamines in the phylum *Apicomplexa* has not been well-characterized [16]. This is the first study that evaluates the effect of putrescine on the *in vitro* proliferation of *B. bigemina*.

The combination of A-DMEM/F12 + M-ITS (2000, 1100, 1.34 mg/L) + Pu (0.1012 mg/L) yielded a higher PPE (14.80%) in treated Bbig-SF cultures than in cultures that added these separately. Similar results have been previously reported for *B. bovis* [20] that suggest this method can be used to stimulate the proliferation of other *Babesia* species. Vega et al. [3] evaluated the F12 nutrient mixture supplemented with 50% of bovine serum in the *in vitro* cultivation of *B. bigemina* and obtained a maximum PPE of 0.200% at 24 h post-initiation. When the components are used independently in the culture medium, they are not able to stimulate the proliferation of the parasite. Bottenstein and Sato [45] reported that there was little or no effect on the proliferation of a rat neuroblastoma cell line when serum-free medium, ITS, progesterone, or Pu were evaluated individually. However, the combination of the elements caused an increase in cell density, a similar result to that obtained with culture medium supplemented with 10% fetal serum. A similar effect on the *in vitro* culture system for the *B. bigemina* strain evaluated in this study is possible.

In a HFPBS, the use of serum-free A-DMEM/F12 medium supplemented with the M-ITS and Pu to stimulate the proliferation of Bbig-SF yielded a higher PPE than cultures added with medium M-199 + bovine serum 40% (33.45% *vs.* 28.75%, respectively). This is similar to the results obtained in the *in vitro* cultivation of *B. bovis* in a HFPBS containing serum-free medium supplemented with Pu-ITS [20].

The HFPBS facilitates low-shear and hydrodynamic conditions and allows exchange of gas and nutrients for intraerythrocytic parasite cultivation. This creates ideal conditions for maintaining *B. bigemina* and *B. bovis* cultured *in vitro*. In another study, the HFPBS was adapted to culture *P. falciparum* at high parasite densities in a high hematocrit (40%) [23]. This culture system provides an excellent source of parasites needed for future studies that will enable a better understanding of phylogenetic relationships of the different species of *Babesia*. This culture system can also contribute to different studies that can involve host cell invasion and pathogen-vector interactions as well as the production of soluble and particulated immunogens to develop live and/or recombinant vaccines.

5. Conclusions

Bovine serum-free A-DMEM/F12 culture medium supplemented with M-ITS and Pu allowed for the continuous *in vitro* proliferation of *B. bigemina*. The elimination of bovine serum from the *in vitro* culture of *B. bigemina* reduced the risk of contamination with adventitious agents and diminished the cost of producing large volumes of *Babesia*-infected erythrocytes. The HFPBS permits a higher density of erythrocytes and a greater harvest of *B. bigemina* infected erythrocytes using a serum-free medium.

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References

[1] R. Bock, L. Jackson, A. de Vos, W. Jorgensen, Babesiosis of cattle, Parasitology 129 (Suppl) (2004) S247–269.

[2] J. Mosqueda, A. Olvera-Ramirez, G. Aguilar-Tipacamu, G.J. Canto, Current advances in detection and treatment of babesiosis, Curr. Med. Chem. 19 (2012) 1504–1518.

[3] C.A. Vega, G.M. Buening, T.J. Green, C.A. Carson, *In vitro* cultivation of *Babesia bigemina*, Am. J. Vet. Res. 46 (1985) 416–420.

[4] F.L. Schuster, Cultivation of *Babesia* and *Babesia*-like blood parasites: agents of an emerging zoonotic disease, Clin. Microbiol. Rev. 15 (2002) 365–373.

[5] R.I. Rodríguez-Vivas, J.A. Trees, Utilización del cultivo *in vitro* de *Babesia bovis* para evaluar la efectividad de babesicidas, Rev. Biomed. 5 (1994) 133–139.

[6] J.A. Álvarez, J.A. Ramos, E.E. Rojas, J.J. Mosqueda, C.A. Vega, A.M. Olvera, J.V. Figueroa, G.J. Cantó, Field challenge of cattle vaccinated with a combined *Babesia bovis* and *Babesia bigemina* frozen immunogen, Ann. N. Y. Acad. Sci. 1026 (2004) 277–283.

[7] C.R. Bautista-Garfias, A.R. Lozano, M.C. Rojas, M.J.A. Álvarez, M.J.V. Figueroa, G.G. Reyes, R. Castañeda-Arriola, B.R. Aguilar-Figueroa, Co-immunization of cattle with a vaccine against babesiosis and *Lactobacillus casei* increases specific IgG1 levels to *Babesia bovis* and *B. bigemina*, Parasitol. Int. 64 (2015) 319–323.

[8] P.J. Price, E.A. Gregory, Relationship between *in vitro* growth promotion and biophysical and biochemical properties of the serum supplement, In Vitro 18 (1982) 576–584.

[9] G. Gstraunthaler, Alternatives to the use of fetal bovine serum: serum-free cell culture, ALTEX 20 (2003) 275–281.

[10] G. Gstraunthaler, The Bologna statement on good cell culture practice (GCCP)-10 Years later, ALTEX 27 (2010) 141–146.

[11] N. Iscove, F. Melchers, Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide reactive B lymphocytes, J. Exp. Med. 147 (1978) 923–933.

[12] J. van der Valk, D. Brunner, K. de Smet, A. Fex Svenningsen, P. Honegger, L.E. Knudsen, T. Lindl, J. Noraberg, A. Price, M.L. Scarino, G. Gstraunthaler, Optimization of chemically defined cell culture media-replacing fetal bovine serum in mammalian *in vitro* methods, Toxicol. In Vitro 24 (2010) 1053–1063.

[13] L. Neves, H.F. Cross, L. Loureiro, A. Akca, M. Hommel, A.J. Trees, Addition of hypoxanthine to culture media allows *in vitro* cultivation of *Babesia bovis* and *Babesia bigemina* at reduced serum concentrations, Parasitology 123 (2001) 357–363.

[14] R.I. Rodríguez-Vivas, F.J. Quiñones-Ávila, G.T. Ramírez-Cruz, D. Cruz, G. Wagner, Aislamiento de una cepa de campo de *Babesia bigemina* (Piroplasma: Babesiidae) y establecimiento del cultivo *in vitro* para la producción de antígenos, Rev. Biol. Trop. 55 (2007) 127–133.

[15] C. Rojas Martínez, R.I. Rodríguez-Vivas, M.J.V. Figueroa, V.K.Y. Acosta, R.E.J. Gutiérrez, M.J.A. Álvarez, *In vitro* culture of *Babesia bovis* in a bovine serum-free culture medium supplemented with insulin, transferrin, and selenite, Exp. Parasitol. 170 (2016) 214–219.

[16] T. Cook, D. Roos, M. Morada, G. Zhu, J. Keithly, J. Feagin, G. Wu, N. Yarlett, Divergent polyamine metabolism in the Apicomplexa, Microbiology 153 (2007) 1123–1130.

[17] O. Heby, S.C. Roberts, B. Ullman, Polyamine biosynthetic enzymes as drug targets in parasitic protozoa, Biochem. Soc. 31 (2003) 415–419.

[18] R. Teng, P.R. Junankar, W.A. Bubb, C. Rae, P. Mercier, K. Kirk, Metabolite profiling of the intraerythrocytic malaria parasite *Plasmodium falciparum* by 1H NMR spectroscopy, NMR Biomed. 22 (2008) 1–11.

[19] L.M. Birkholtz, M. Williams, J. Niemand, A.I. Louw, L. Persson, O. Heby, Polyamine homoeostasis as a drug target in pathogenic protozoa: peculiarities and possibilities, Biochem. J. 438 (2011) 229–244.

[20] C. Rojas-Martínez, R.I. Rodríguez-Vivas, M.J.V. Figueroa, V.K.Y. Acosta, R.E.J. Gutiérrez, M.J.A. Álvarez, Putrescine: essential factor for *in vitro* proliferation of *Babesia bovis*, Exp. Parasitol. 175 (2017) 79–84.

[21] J.V. Figueroa, L.P. Chieves, G.S. Johnson, G.M. Buening, Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood, Vet. Parasitol. 50 (1993) 69–81.

[22] C. Rojas, J.V. Figueroa, A. Alvarado, P. Mejía, J.J. Mosqueda, A. Falcón, C.A. Vega, A. Álvarez, Bovine babesiosis live vaccine production: use of gamma irradiation on the substrate, Ann. N. Y. Acad. Sci. 1081 (2006) 405–416.

[23] P. Preechapornkul, K. Chotivanich, M. Imwong, A.M. Dondorp, S.J. Lee, N.P. Day, N.J. White, S. Pukrittayakamee, Optimizing the culture of *Plasmodium falciparum* in hollow fiber bioreactors, Med. Public Health 41 (2010) 761–769.

[24] B.M. Monroy, O.G. Romero, T.R. Aboytes, M.J.A. Álvarez, A.J.G. Canto, C.A. Vega, Establecimiento en México del cultivo *in vitro* de *Babesia bigemina*, Téc. Pec. Méx. 25 (1987) 141–150.

[25] D. Barnes, G. Sato, Methods for growth of cultured cells in serum-free medium, Anal. Biochem. 102 (1980) 255–270.

[26] D. Barnes, G. Sato, Serum-free cell culture: a unifying approach, Cell 22 (1980) 649–655.

[27] B. Tuvshintulga, M. AbouLaila, B. Davaasuren, A. Ishiyama, T. Sivakumar, N. Yokoyama, M. Iwatsuki, K. Otoguro, S. Ōmura, I. Igarashi, Clofazimine inhibits the growth of *Babesia* and *Theileria* parasites *in vitro* and *in vivo*, Antimicrob. Agents Chemother. 60 (2016) 2739–2746.

[28] K. Brayton, A. Lau, D. Herndon, L. Hannick, L. Kappmeyer, S. Berens, S. Bidwell, W. Brown, J. Crabtree, D. Fadrosh, T. Feldblum, H. Forberger, B. Haas, J. Howell, H. Khouri, H. Koo, D. Mann, J. Norimine, I.T. Paulsen, D. Radune, Q. Ren, R. Smith, C. Suarez, O. White, J. Wortman, D. Knowles, T. McElwain, V.M. Nene, Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa, PLoS Pathog. 3 (2007) 1401–1413.

[29] H. Asahi, T. Kanazawa, Continuous cultivation of intraerythrocytic *Plasmodium falciparum* in a serum-free medium with the use of a growth promoting factor, Parasitology 109 (1994) 397–401.

[30] H. Asahi, T. Kanazawa, Y. Kajihara, K. Takahashi, T. Takahashi, Hypoxanthine: a low molecular weight factor essential for growth of erythrocytic *Plasmodium falciparum* in a serum-free medium, Parasitology 113 (1996) 19–23.

[31] E. Zweygarth, C.J. van Niekerk, D.T. De Waal, Continuous *in vitro* cultivation of *Babesia caballi* in serum-free medium, Parasitol. Res. 85 (1999) 413–416.

[32] E. Sheldon Broedel Jr., The Case for Serum-free Media, vol. 1, Athena Environmental Sciences, Inc., Baltimore, MD, 2003, pp. 56–58.

[33] M.D. Jensen, M. Conley, L.D. Helstowski, Culture of *Plasmodium falciparum*. The role of pH, glucose and lactate, J. Parasitol. 69 (1983) 1060–1067.

[34] E.G. Ball, R.W. McKee, C.B. Anfinsen, W.O. Cruz, Q.M. Geiman, Studies on malarial parasites: IX.Chemical and metabolic changes during growth and multiplication *in vivo* and *in vitro*, J. Biol. Chem. 175 (1948) 547–571.

[35] S.E. Francis, D.J. Sullivan Jr., D.E. Goldberg, Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*, Annu. Rev. Microbiol. 51 (1997) 97–123.

[36] J. Brozmanová, D. Mániková, V. Vlčková, M. Chovanec, Selenium: a double-edged sword for defense and offence in cancer, Arch. Toxicol. 84 (2010) 919–938.

[37] S. Müller, T.W. Gilberger, Z. Krnajski, K. Lüersen, S. Meierjohann, R.D. Walter, Thioredoxin and glutathione system of malaria parasite *Plasmodium falciparum*, Protoplasma 217 (2001) 43–49.

[38] E. Jortzik, K. Becker, Thioredoxin and glutathione systems in *Plasmodium falciparum*, Int. J. Med. Microbiol. 302 (2012) 187–194.

[39] B. Gamain, J. Arnaud, A. Favier, D. Camus, D. Dive, C. Slomianny, Increase in glutathione peroxidase activity in malaria parasite after selenium supplementation, Free Radic. Biol. Med. 21 (1996) 559–565.

[40] N. Taguchi, T. Hatabu, H. Yamaguchi, M. Suzuki, K. Sato, S. Kano, *Plasmodium falciparum*: selenium-induced cytotoxicity to *P. falciparum*, Exp. Parasitol. 106 (2004) 50–55.

[41] E.W. Suradji, T. Hatabu, K. Kobayashi, C. Yamazaki, R. Abdulah, M. Nakazawa, J. Nakajima-Shimada, H. Koyama, Selenium-induced apoptosis-like cell death in *Plasmodium falciparum*, Parasitology 138 (2011) 1852–1862.

[42] I.B. Müller, R. Das Gupta, K. Lüersen, C. Wrenger, R.D. Walter, Assessing the polyamine metabolism of *Plasmodium falciparum* as chemotherapeutic target, Mol. Biochem. Parasitol. 160 (2008) 1–7.

[43] C.M. Rzepczyk, A.J. Saul, A. Ferrante, Polyamine oxidase-mediated intraerythrocytic killing of *Plasmodium falciparum*: evidence against the role of reactive oxygen metabolites, Infect. Immun. 43 (1984) 238–244.

[44] K. Clark, J. Niemand, S. Reeksting, S. Smit, A. Brummelen, M. Williams, A. Louw, L. Birkholtz, Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*, Amino Acids 38 (2010) 633–644.

[45] J.E. Bottenstein, G.H. Sato, Growth of a rat neuroblastoma cell line in serum-free supplemented medium, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 514–517.