New Approach To *In-Vitro* Propagation Of *Babesia Bovis* In Low Serum Content

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ABSTRACT

In-vitro propagation of *B. bovis* using microaerophilous stationary phase (MASP) were investigated. Medium199 (Med_{199}) containing 40% bovine serum showed good propagation of the parasites represented by the percent of infected red blood corpuscles. Reduction in bovine serum content in Med_{199} to 20% or less resulted in lower growth of the parasite.

LH-1 medium containing 20% bovine serum was gradually replaced Med_{199} containing 40% serum. After ten serial passages, the parasite was adapted for growth in the LH-1 medium with 20% serum. Other successful trials to reduce serum content in the culture to 10% were done. There were no clear differences in the percent of infected RBCs when Med199 containing 40% serum, LH-1 medium containing 20% bovine serum or LH-1 medium containing 10% bovine serum.

The viability of cryopreserved parasites which had been stored in liquid nitrogen was realized encountered after re-cultivation in MASP under optimum conditions in 5% CO_2 atmosphere.

INTRODUCTION

Babesia (B.) bovis is a haemoprotozoan which causes bovine babesioisis (Texas fever), a heamolytic tick-born disease. The disease is of high economic importance in livestock production animals *(1,2)*. Before 1978, several unsuccessful attempts to *in-vitro* propagation of *Babesia* organisms have been made to produce antigens for serological tests and immunogens for disease control *(3,4)*.

The development of continuous *in-vitro* cultures of *B. bovis* using microaerophilous stationary phase (MASP) provided reproducible sources of tissue culture live attenuated vaccine, dead antigens and soluble derived exoantigens *(5)*. Exoantigens are soluble proteins naturally released into the supernatant medium of cultures of these organisms.

A major problem of continuous *in-vitro* cultures of *B. bovis* in MASP is the high concentration of bovine serum protein requirement (40%) which provided only from a full susceptible calf (a haemo-parasite free calf kept ticks free yard). Not all babesia free young or adult cattle provide serum that supports growth potential donors need to be screened in on-going culture for growth supporting capabilities *(6)*.

The aim of the present study was to fulfill the following itemes : 1- detecting the most suitable conditions for optimizing the growth of *B. bovis* (Egyptian strain as a source of immunogens. 2- Gradually replacing conventional medium (medium 199 with 40% normal adult bovine serum) with HL-1 medium containing low serum content. 3- storing in liquid nitrogen and retrieving the viability of the cryopreserved parasites.

MATERIAL AND METHODS

1. **Donor animal:** a haemo-parasite free calf, as confirmed by Giemsa-staind blood smears and Indirect Immuno-fluorecent Antibody technique (IFA) using *B. bovis* specific IgG *(7,8)*., was kept under hygienic conditions in tick free yard. This calf was used for collection of serum and defibrinated blood for uninfected erythrocytes. The erythrocytes were prepared as a 50% suspension in Puck's

saline solution (PSS) containing 10% glucose.

2.Isolation of *B. bovis* (Egyptian strain): The strain was isolated from blood of naturally infected cattle at Damanhour, Behira, Egypt and identified microscopically beside Indirect Immuno-flurecent Antibody technique (IFA) using *B. bovis* specific IgG *(7,8)*. The blood of infected cattle was collected aseptically into a sterile flask containing sterile glass beads and defibrinated just after collection by vigorous swirling of the glass beads in the flask. The defibrinated blood was transferred to centrifuge tubes and centrifuged at 500 g for 30 min using a refrigerated centrifuge. The plasma and white blood corpuscles (WBCs) were removed. The RBCs were washed 3 times in sterile PSS buffer pH 7.4 and sedimented by centrifugation then withdrawn from the bottom of the tube and used in the cultivation of the parasite.

3.Media and supplement for *B. bovis* cultivation

1-Media: The following two mediea were prepared

a-Medium 199 according to the manufacture procedures (Gibico).

b-LH-1 medium was prepared according to the manufacture procedures (Hycor Biomedical inc).

2-Buffer: TES (N-tris-methyl2-aminoethan-sulfonic acid buffer) at conc. Of 20 mM of the prepared media

3.Bovine serum 10-40% bovine serum was added to the media.

4.Antibiotics: a mixture of 100 ug streptomycin, 100 IU penicillin was mixed per each ml of the prepared medium.

The prepared medium was sterilized through 0.45-micron filter and then refrigerated until used.

4. In vitro* Cultivation of *B. bovis

The cultivation was done according to the method described previously *(5)* with the modification *(9)*, briefly, the parasite of infected RBCs were added to defibrinated uninfected RBCs (from the donor cow) at 1:3 ratio by volume respectively. The PCV of RBCs was 10% while the prepared medium was 90%. The prepared medium was supplemented with 40% bovine serum, 20 mM TES as buffer and a mixture of 100 ug streptomycin, 100 IU penicillin were mixed per ml of the prepared medium. The cultures were incubated at 37°C in a humidified 5% CO_2 incubator. Feeding was conducted every 24 hours (hrs) intervals by removing the supernatant media and replaced with fresh prepared medium. The culture was subcultured every 48 hours for 40 passages.

5.Subculture of the parasites in 24-wells cultured plate *(9)*

A volume of 1.0 ml of fresh prepared medium was put in each new well. One ml of the old supernatant of each cultured well was removed. A thin smear of cells was made to detect parasiteamia %. The cell layer in the old well was suspended in 1 ml of fresh medium. A volume of 0.25 ml of this suspension (infected RBCS) were added to each new well, then 0.15 ml of the 50% suspension of uninfected RBCs collected from donor animal was added to each new well. The plate was incubated in a CO_2 incubator. Feeding at 24 hrs intervals was conducted.

***6.* Feeding of 24 well plate culture**

An aliquot of 1 ml of supernatant medium of each well was removed without disturbing the cell layer. A thin smear was made from the sediment and stained with Giemsa to estimate the percent of infected RBCs after 24 hrs. The removed supernatant was replaced with an equivalent volume of new medium. The plate was incubated in a CO_2 incubator.

7.Estimation the percent of B. bovis infected RBCs

Ten microlitres of cells were withdrawn aseptically from the established culture. The percent of infected RBCs of the culture was evaluated by microscopical examination of thin smeared stained with Giemsa stain. The

percentage of infected RBCs was calculated in at least 20 field (x1000) in Giemsa stain blood films.

8. Growth of *B. bovis* in LH-1 medium containing 20% serum.

At passages 14, the parasite was cultivated in a mixture of med 199 and LH1. Med. 199 containing 40% serum was gradually replaced by LH-1 containing 20% serum (25% in passage 15, 50% in passage 16, & 75% in passage 17) then 2 succssieve passages in 100% LH-1 containing 20% serum

9. Growth of *B. bovis* in LH-1 medium containing 10% serum

At passages 25, the parasite was transferred into new plate and cultivated in LH-1 containing 15% serum for 2 successive passages. Then the parasite was propagated in LH-1 medium containing 10% serum for 3 successive passages was done.

10.* Cryopreservation of cultivated *Babesia bovis (10)

The cultivated cells were re-suspended in its supernatant. The suspension was centrifuged at 500 g for 20 min at 4 °C. The pellet was gently re-suspended in 3 M dimethyl sulphoxide solution. Aliquots of 0.5 ml of this suspension were loaded in cryovial tubes. The cryovial tubes were put in an ethanol bath at – 70 °C and left overnight, then the tubes were stored in a liquid nitrogen tank.

11.* Retrieve of the cryopreserved *B. bovis* stabilates *(9).

The content of cryovial was thawed rapidly after removal from liquid nitrogen in 37°C water bath. Aliquots of 0.25 ml of the stabilate (infected RBCs) were put into a well of 24-wells tissue culture plate containing 1.0 ml of Med199 and 0.1 ml of uninfected RBCs suspension. The culture was incubated at 37 °C in a 5% CO_2 The cultures were fed at 24 hrs interval. The first subculture was done when 7 - 10 paired *Babesia* infected RBCs were detected in a 100 X-field on a Giemsa stained smear

RESULTS

After 5 passages, *Babesia bovis* was cultivated in medium 199 with 40% bovine serum and incubated at 37 °C in 5% Co_2, humidified gas in cultured wells, the percent of infected RBCsFF:\ WINDOWS\ hinhem. scr in wells increased to 2.1% and 4.0 % after 24 and 48 hr respectively.

The effect of three serum concentrations on multiplication rate of *B. bovis* was tested during passages numbers (6-10) in Med199 containing 10%, 20% or 40% bovine serum showed differences on *B. bovis* multiplication. The mean percent of infected RBCs in culture containing 40% serum was increased to 4.3% after 48 hours, in passage number 10. Reduction of serum to 20% resulted in lowering the propagated rate of parasite, as the mean percent of *B. bovis* infected RBCs was decreased to 2.9% in passage number 10. In case of using 10% serum, *B. bovis* infected RBCs was also decreased to 2.0 % only in passage number 10 (Table 1).

Continuous subculture of *B. bovis*, at 5% CO_2 atmosphere, resulted in gradually increasing the multiplication rate. By passage 10, the mean percent of infected RBCs were 2.1% after 24 hrs and 4.0% after 48 hrs, while in passage 20, it was 2.5% and 6.1% after 24 hrs and 48 hrs respectively. By passage 30, the mean percent of infected RBCs were 5.2% and 9.8% after 24 hrs and 48 hrs respectively. The mean percent of infected RBCs at passage 40 were 6.1% after 24 hrs and 12.8% after 48 hrs. (Table 2 and Fig.1).

The efficiency of 2 the common used media (Med_{199} and LH-1 supplemented with 20 mM TES as a buffer) were evaluated in cultivation of *B. bovis* . Med199 contained 40% bovine serum yielded lower growth rat of *B. bovis* than LH-1medium contained 20% bovine serum. The mean percent of infected RBCs was increased gradually from 6.1% at passage No. 20 to 7.6% after 48 hrs of passage No. 24 while using LH-1 contained 20% serum, it increased from 6.3% to 8.1% after the same successive passages (Table 3).

The effect of the three serum concentrations on multiplication rate of *B. bovis* was determined during passages numbers 30 -34. LH-1 medium containing either 20% or 10% bovine serum showed slight difference on *B. bovis* multiplication, whereas the mean percent of infected RBCs was 11.4% or 11.0% respectively after 48hours in passage number 34 (Table 4 and Fig.1). Reduction of serum to 5% resulted in lowering the multiplying rate of the parasite, as the mean percent of *B. bovis* infected RBCs was 5.9% after 24 hours in passage number 34. Cultivation of the parasite in Med_{199} containing 40% serum was used as positive control. The mean percent of infected RBCs was 11.2% after 48hours in passage number 34 (Table 4 and Fig. 1).

Table 1. The effect of serum content on the *in vitro* growth of *B. bovis* in Med199.

Number of passages	Mean percent of *B. bovis* infected RBCs								
	Serum concentration								
	40%			20%			10%		
	At 0 hr	After 24 hr	After 48 hr	At 0 hr	After 24 hr	After 48 hr	At 0 hr	After 24 hr	After 48 hr
6	0.975	2.0	4.0	0.975	1.4	3.1	0.975	1.1	2.3
7	1.00	2.0	4.0	0.775	1.6	3.0	0.575	1.0	2.1
8	1.00	2.0	4.2	0.750	1.4	2.6	0.525	0.9	2.0
9	1.05	2.1	4.2	0.65	1.4	2.7	0.500	1.0	2.0
10	1.05	2.1	4.3	0.675	1.5	2.9	0.500	0.9	2.0

hr = hours

Table 2.The effect serial continuous passages of *B. bovis* in Med199 containing 40% serum.

Number of passages	Mean percent of *B. bovis* infected RBCs Medium 199 containing 40% serum.		
	At 0 hr	After 24 hr	After 48 hr
10	1.05	2.1	4.0
20	1.5	2.5	6.1
30	2.5	5.2	9.8
40	3.2	6.1	12.8

hr = hours

Table 3. The *in vitro* propagation of *B. bovis* in two types of media.

Number of passages	Mean percent of *B. bovis* infected RBCs					
	Med199 containing 40% serum			LH-1 Medium containing 20% serum		
	At 0 hr	After 24 hrs	After 48 hrs	At 0 hr	After 24 hrs	After 48 hrs
20	1.50	3.60	6.10	1.50	3.70	6.30
21	1.52	3.65	6.40	1.57	3.85	6.50
22	1.60	3.80	6.90	1.62	3.90	7.30
23	1.72	3.90	7.20	1.82	4.00	7.80
24	1.80	4.00	7.60	1.95	4.30	8.10

Table 4. The effect of serum content in the *in vitro* growth of *B. bovis* in LH-1 medium and Med199 as control.

Number of passages	Med199 containing 40% serum			LH-1 med containing 20% serum			LH-1 med containing 10% serum		
	At 0 hr	After 24 hr	After 48 hr	At 0 hr	After 24 hr	After 48 hr	At 0 hr	After 24 hr	After 48 hr
30	2.45	5.10	9.8	2.50	5.3	10.2	2.50	5.2	10.0
31	2.45	5.20	10.2	2.55	5.3	10.2	2.50	5.10	10.0
32	2.55	5.15	10.2	2.55	5.35	10.4	2.50	5.3	10.2
33	2.55	5.40	10.7	2.60	5.70	10.8	2.55	5.4	10.5
34	2.67	5.90	11.2	2.70	5.90	11.4	2.62	5.50	11.0

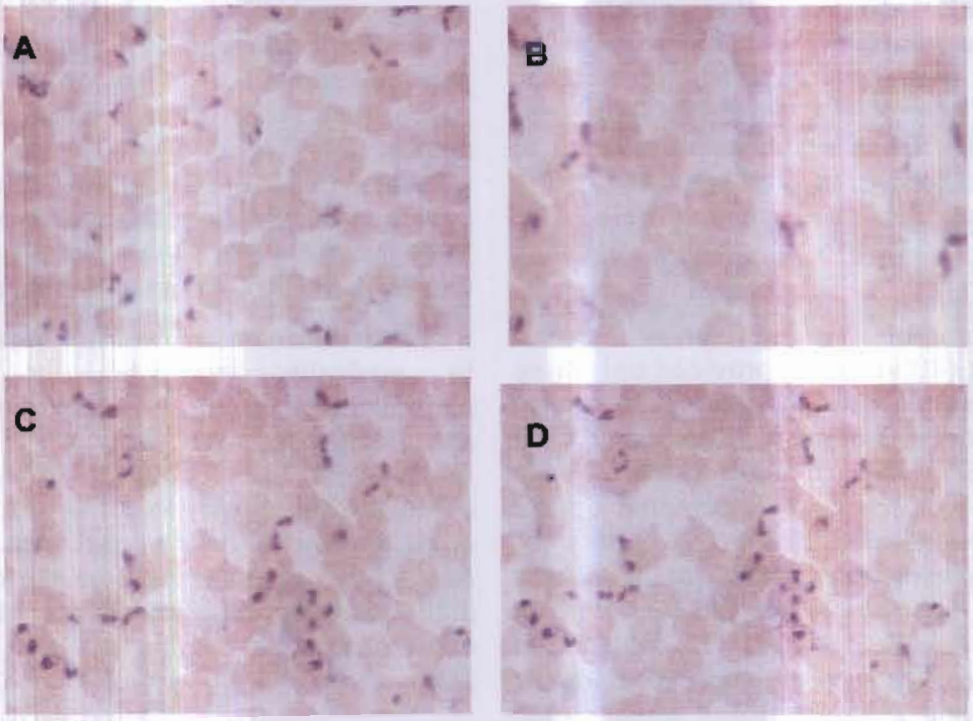

Fig. 1, *B. Bovis* infected RBCs yielded from *in-vitro* cultivation of the parasite using MASP system in different media and serum concentration.

A : *B. bovis* cultivated in Med199 containing 40% serum

B : *B. bovis* cultivated in Med199 containing 20% serum

C and D: *B. bovis* cultivated in LH-1 med containing 10% serum

DISCUSSION

The development of continuous *in vitro* cultures of *B. bovis* has greatly facilitated laboratory studies of babesiosis ***(4)***, including production of immunogens as soluble derived exoantigens ***(11)***, live attenuated vaccine and merozoite antigens ***(12)***.

The primary aim of the present study was to optimize growth conditions of *in-vitro* propagation of *B. bovis* required for the production of different immunogens. Type of medium, buffer and concentration of bovine serum are the most common factors of *in-vitro* cultivation of *B. bovis*, therefore 2 types of common media and different concentration of serum were prepared and investigated its effects in propagation of *B. bovis*. The majority of researchers, as ***(3, 9,13-15)*** used 40% or 50% bovine serum in propagation of *B. bovis.*, in Med_{199} and incubated at 37 °C in 5% Co_2, humidified gas in cultured wells.

A major problem of continuous *in-vitro* cultures of *B. bovis* in MASP is the high concentration of bovine serum protein requirement (40%) which provided only from a full susceptible calf (a haemo-parasite free calf kept ticks free yard). Moreover not all *babesia* free young or adult cattle provide serum that supports growth potential donors need to be screened in on-going culture for growth supporting capabilities ***(6)***. It follows that setting up cultures for the first time in babesiosis endemic area. It has been recommended the use of lyophilized bovine serum as a substitute for frozen serum in the cultivation of *B. bovis* ***(6)***

In trials to reduce the amount of bovine serum in med199 used in cultivation of *B. bovis* from 40% to 30% or 20%, the studies revealed that 30% serum in media resulted in a slight reduction in the mean percent of infected RBCs (from 5% to 4.8%), while 20% serum decreased the percent of infected RBCs to 2.9% under the same conditions during 5 successive passages (Table 1). These data agreed with the recommendation of ***(9)*** that *B. bovis* was cultivated in $Med_{199}$with 40% bovine serum and incubated at 37°C in 5% Co_2, humidified gas in cultured wells.

Successful trials to study the reduction effect of serum concentrations on multiplication rate of *B. bovis* were tested using LH-1 med. during passages numbers 30 -34. LH-1medium containing either 20% or 10% bovine serum showed slight difference on *B. bovis* multiplication, whereas the mean percent of infected RBCs was 11.4% or 11.0% after 48hours in passage number 34 respectively (Table 4).. Reduction of serum to 5% resulted in lowering the growth rate of parasite, as the mean percent of *B. bovis* infected RBCs was 5.9% after 24 hours in passage number 34 . Cultivation of the parasite in Med199 containing 40% serum was used as positive control. The mean percent of infected RBCs was 11.2% after 48hours in passage number 34 (Table 4). *B. bovis* could be cultivated in Med_{199} using 50% replacement of bovine serum with human serum type (O) ***(16)***. Fifty percentage of bovine serum was better than 75% or 100% of human serum ***(16)***.

Continuous subculture of *B. bovis*, resulted in gradually increasing the rate of multiplication during 40 successive passages and increasing the amount of parasitic immunogens in culture. So continuous subculture for preparation of live attenuated *B. bovis* vaccine after 75 serial passages was used ***(12)***. In the same time the efficacy of high passages in production of babesial exoantigens as a vaccine has been confirmed ***(15)***.

The viability of cryopreserved parasites which had been stored in liquid nitrogen was confirmed after re-cultivation of parasite in Med199 with 40 % serum incubated in 5% CO2. This important observation parallels with the study which demonstrated that the highest infectivity rate (46%) was obtained by using 3M DMSO ***(17)***.

In conclusion, the optimum *in-vitro* growth of *B. bovis* in MASP system was realized using either Med199 containing 40% bovine serum or LH-1 medium containing 20 or 10% bovine serum. Continuous subculture

of *B. bovis*, resulted in gradually increasing the rate of parasite multiplication. The viability of cryopreserved parasites which had been stored in liquid nitrogen has been proved.

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الملخص العربى

محاولات جديدة لإكثار طفيل البابيزيا بوفيز معملياً في أقل كمية سيرم ممكنة

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تم إكثار طفيل البابيزيا بوفيز معملياً باستخدام الميكروايروفريس ستشنري فيز Microaerophilous stationary phase و قد وجد ان الوسط الغذائي M199 الذي يحتوي علي ٤٠% سيرم عجول مناسب لنمو الطفيل بحالة جيدة ممثلاً في نسبة إصابة الطفيل في كرات الدم الحمراء و عندما اختزلت كمية السيرم الي ٢٠ % أو أقل تسبب ذلك في إنخفاض نسبة الإصابة بالطفيل و عندما استبدل الوسط الغذائي LH-1 و الذي يحتوي علي ٢٠ % سيرم تدريجياً تعود الطفيل علي النمو في الوسط الجديد (LH-1) خلال عشرة تمريرات.

و قد أمكن نمو الطفيل علي نفس الوسط الغذائي (LH-1) مع اختزال كمية السيرم إلي ١٠% و لم يوجد فروق واضحة بين نسبة الإصابة بالطفيل فى كرات الدم الحمراء فى الاوساط الغذائية المختلفة (M_{199} تحتوى على ٤٠% سيرم و LH-1 تحتوى على ٢٠% سيرم و LH-1 تحتوى على ١٠% سيرم).

وقد تم التأكد من حيوية و نمو الطفيل بعد حفظه في السائل النيتروجيني و إعادة زرعة من جديد بإستخدام الميكروايروفريس ستشنري فيز Microaerophilous stationary phase تحت الظروف المناسبة و ٥ % ثاني أكسيد الكربون.