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RESEARCH COMMUNICATION

In vitro cultivation of *Babesia occultans*

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ABSTRACT

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Babesia occultans, the causative agent of a benign form of cattle babesiosis in South Africa, was continuously cultivated in microaerophilous stationary-phase culture. A modified medium, 199, supplemented with either 40% (v/v) bovine or 40% (v/v) horse serum, was used. Cultures were initiated in a humidified atmosphere containing 2% O_2, 5% CO_2, and 93% N_2. The highest percentage of parasitized erythrocytes (PPE) reached 4,5% in horse-serum- and 2,4% in bovine-serum-supplemented medium. Parasite suspensions were cryopreserved and successfully resuscitated.

Keywords: *In vitro* cultivation, *Babesia occultans*, bovine babesiosis, South Africa

INTRODUCTION

In a study by Thomas & Mason (1981), "large merozoites" of a *Babesia* sp. were found in the haemolymph of engorged adult female *Hyalomma marginatum rufipes* ticks. This tick species was not previously known to be a vector of any *Babesia* sp. The same authors showed that the piroplasm stages and the kinetes of the parasite ranged in size between those of *B. bovis* and *B. bigemina*. It was subsequently shown that this new parasite, *B. occultans* (Gray & De Vos 1981), was highly infective for *H. m. rufipes*, but not for *Boophilus microplus*, the vector for *B. bovis* and *B. bigemina*. The present study describes the establishment of *B. occultans* in microaerophilous stationary-phase culture (Levy & Ristic 1980).

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MATERIALS AND METHODS

Babesia occultans isolate

The isolation of *B. occultans* has been described in detail elsewhere (Gray & de Vos 1981; Thomas & Mason 1981). Briefly, ticks were collected on a farm near Ellisras in the Northern Transvaal (South Africa). The progeny of these ticks were used to transmit the parasite to splenectomized cattle. Blood stabilates were prepared with 8% (v/v) dimethylsulphoxide (DMSO) as a cryoprotectant, and stored in the gas phase of a liquid-nitrogen container. At the time of stabilate preparation, the percentage of parasitized erythrocytes (PPE) was approximately 0,2%.

Experimental animal

A 1-year-old splenectomized ox, raised under tick-free conditions and free from blood protozoa, was injected intravenously with 4,5 mℓ of a *B. occultans* blood stabilate (Gray & De Vos 1981). Blood smears were prepared daily and examined for the presence

of babesia. In addition, the packed-cell volume (PCV) and rectal temperature of the steer were determined daily. Starting 2 d before stabilate inoculation, the animal was injected on alternate days with prednisolone 1 mg kg^{-1} (Deltacortril, Pfizer), until the parasitaemia started to drop. The dose was then halved each day to prevent an iatrogenic adrenocortical insufficiency by the sudden withdrawal of the drug. The animal was kept under tick-free conditions during the whole experiment.

Culture medium

The medium used, consisted of medium 199 (with Hanks' salts) with either 40% bovine serum or 40% horse serum, buffered with 20 mM of TAPSO (3-[N-tris-{hydroxymethyl}methyl-amino]-2-hydroxypropane-sulfonic acid; Sigma, St. Louis, Mo., USA; Goff & Yunker 1988) and supplemented with 100 IU/mℓ of penicillin and 100 µg/mℓ of streptomycin.

Culture initiation and maintenance

For initiation of cultures, blood from the infected ox was collected by venipuncture into sterile vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The blood cells were washed three times by centrifugation (1 000 x *g*, 10 min, room temperature) and resuspended in modified Vega y Martinez (mVYM) phosphate-buffered saline solution (Zweygarth, Just & De Waal 1995). After each centrifugation, the white-blood cells were removed from the bovine red-blood cells (BRBC) by the use of an Eppendorf pipette. After the last wash, 100 µℓ of the packed RBC suspension and 900 µℓ of medium were pipetted into a 24-well culture plate. The plates were initially incubated in an atmosphere of 2% O_2; 5% CO_2 and 93% N_2 at 37°C. The culture plates were transferred to a 5-%-CO_2-in-air atmosphere when the PPE was between 0,5 and 1%.

In another experiment, cultures were initiated directly in a 5-%-CO_2-in-air atmosphere. Medium was replaced daily, 20 µℓ of fresh, uninfected BRBC was added once a week until the cultures were subcultured. The washing procedure of BRBC from an uninfected donor animal, was the same as that described above.

Estimation of parasite growth

The PPE of cultures was determined by counting five fields of approximately 500 BRBC, each on a thin blood film stained with either 10% Giemsa for 35 min or with a quick-staining kit (RapiDiff, Clinical Sciences Diagnostics, Booysens, South Africa).

Preparation and retrieval of stabilates

For cryopreservation, erythrocytes from cultures were collected and centrifuged (1000 x *g*, 10 min, room temperature) and a 40% (v/v) suspension of erythrocytes in fresh complete culture medium was prepared. Equal volumes of cell suspension and pre-cooled 4% (v/v) DMSO in complete medium were mixed, and 800 µℓ aliquots of the mixture were then transferred into 1-mℓ cryotubes and kept at –70°C overnight in a polystyrene container. The following day, they were transferred for long-term storage into the gas phase of a liquid-nitrogen container.

Resuscitation was done by thawing two stabilate vials in a water bath at 37°C and the contents were diluted in 40 mℓ of mVYM solution. After centrifugation (3,000 x *g*, 10 min, 4°C) the pellet was resuspended in 2 mℓ of complete culture medium with 10% (v/v) fresh, unparasitized erythrocytes; this was divided into two wells of a 24-well culture plate. The plate was then incubated at 37°C in the oxygen-reduced gas mixture as described above.

RESULTS

Parasites were detected in Giemsa-stained thin blood smears of the experimental animal between days 5 and 14, after stabilate injection. The parasitaemia was generally low, and a PPE of more than 1,8% was observed only on days 10 and 11 (19% and 16%, respectively). On days 12 and 13, the parasitaemia dropped to 0,8% and < 0,01%, respectively. After day 14, parasites were no longer detected. The rectal temperature of the steer ranged between 40,0 and 41,3°C on days 9, 10 and 11. The PCV dropped from 35% pre-infection to 19%, on day 11 post infection. The infection of the steer with *B. occultans* did not produce severe clinical symptoms. The course of infection was very mild, similar to that described by Thomas & Mason (1981). After 14 d, parasites disappeared rapidly from the blood, and therefore treatment was not necessary. This is in contrast to observations made of other bovine *Babesia* species where splenectomy exacerbated clinical reactions in the host animal (Riek 1968).

Blood collected on day 6 was adjusted to a PPE of 0,6% before being used for culture initiation. Incubation was done with horse-serum-supplemented medium in the oxygen-reduced gas mixture. After an initial increase in the PPE, there was a steady decrease, until they were only rarely detected in stained smears. From day 14 onwards, the PPE increased slowly and subcultures were first made on day 19, when a PPE of 0,8% was recorded. Subsequently, subcultures were made every other day. On day 35, the PPE reached 4% in subcultures. The highest PPE obtained at any stage, was 4,5%. The parasites were propagated for ten weeks before they were cryopreserved. In parallel experiments, bovine-serum-supplemented medium did not support culture initiation.

When cultures were initiated in an atmosphere of 5 % CO_2 in air, prior to the reduction of the parasite

concentration, the PPE in culture initially increased from 1,8% to 2,4% (from 1,8% to 3,2% in horse-serum-supplemented medium), but when cultures were split at a ratio of 1:2 (v/v), parasites ceased to grow. Using another batch of bovine serum, however, and the oxygen-reduced gas mixture, it was possible to initiate cultures from blood obtained on day 13 of infection, when the PPE was below 0,01%.

The parasites remained viable and resumed growth after cryopreservation. They were detected in blood smears on day 4 after resuscitation. Subcultures were done on day 7, when the PPE reached approximately 4%.

CONCLUSIONS

The experiments showed that *B. occultans* can be initiated and propagated *in vitro* under conditions similar to those described for *B. bovis* (Rodriguez, Buening, Green & Carson 1983) and *B. bigemina* (Vega, Buening, Green & Carson 1985). Bovine and horse serum are both suitable for the cultivation of *B. occultans*, provided a good serum batch is chosen. Similar results were described for *B. bovis* and *B. bigemina* cultures (Fish, Pipano, Shkap & Frank 1993) where bovine serum could be replaced by horse serum without negatively influencing parasite growth. In our experiments, higher PPEs were produced in horse serum than in bovine serum.

Research is currently under way, which aims to increase the PPE in the culture system. To date, culture-derived parasites have been successfully used by us as antigen for the indirect immunofluorescence antibody test.

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