RESEARCH COMMUNICATION

In vitro cultivation of a *Babesia* sp. from cattle in South Africa

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ABSTRACT

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A South African *Babesia* sp. of cattle which is as yet unclassified, was continuously cultivated in microaerophilous stationary-phase culture. The parasites were resuscitated from a blood stabilate stored in liquid nitrogen. A modified HL-1 medium supplemented with either horse or bovine serum was used. Cultures were initiated in a humidified atmosphere containing 2 % O_2, 5% CO_2 and 93 % N_2 at 37 °C. Parasites were detected on Giemsa-stained smears after 2 d in culture. On day 4, the cultures were split at a ratio of 1:2 (v/v) and transferred into a humidified atmosphere of 5% CO_2 in air. Starting from day 6, subcultures were made daily at a ratio of 1:4 (v/v). The percentage of parasitized erythrocytes ranged from 2–5%. Addition of purine bases (hypoxanthine, adenine, adenosine or guanosine) was essential for the continuous propagation of the parasites when bovine, but not horse serum, was used for medium supplementation.

Keywords: *In vitro,* cultivation, *Babesia,* cattle, bovine, South Africa

INTRODUCTION

Babesiosis is a major tick-transmitted protozoal disease of cattle. In South Africa, *B. bigemina* and *B. bovis* are the two economically important agents of bovine babesiosis. Both species are transmitted by *Boophilus microplus; B. bigemina* is also transmitted by *Boophilus decoloratus* (Potgieter 1977). Gray & De Vos (1981) described another *Babesia* of cattle which they isolated in South Africa. This parasite, named *B. occultans*, causes a mild disease and is transmitted transovarially by *Hyalomma marginatum rufipes,* a tick species which transmits neither *B. bovis* nor *B. bigemina*. The fourth bovine *Babesia* described in South Africa is as yet unclassified. It was not transmissible by *B. microplus* and *H. m. rufipes*, but by *H. truncatum* (De Waal, Potgieter, Combrink & Mason 1990).

The present paper describes the culture initiation and continuous *in vitro* propagation of *Babesia* sp.

MATERIALS AND METHODS

Babesia sp. isolate

The parasite was isolated at Kaalplaas Farm (28° 08´E, 25°38´S), Onderstepoort Veterinary Institute (South Africa) (De Waal *et al.* 1990). The stabilate from which the cultures were initiated was cryopreserved according to the method of De Vos, Combrink

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& Bessenger (1982). When the stabilate was prepared, the percentage of parasitized erythrocytes (PPE) was 0,002%.

Culture medium

The medium, referred to as complete medium, consisted of HL-1 medium (Hycor Biomedical Inc., Portland, Maine, USA) with either 20% horse or bovine serum, buffered with 15 mM HEPES supplemented with 2 mM l-glutamine, 0,2 mM hypoxanthine, 200 IU penicillin/mℓ and 200 µg streptomycin/mℓ. After 5 d, the concentration of the antibiotics in the medium was halved. Some media compositions were altered by omitting hypoxanthine or by replacing it with 0,2 mM adenine, adenosine or guanosine.

Culture initiation and maintenance

A vial of stabilate was thawed in a water bath at 37 °C and the contents were diluted into 20 mℓ of a modified Vega y Martinez phosphate-buffered saline solution (mVYM) (Zweygarth, Just & De Waal 1995). After centrifugation (2 000 x *g*, 10 min, 4 °C), the pellet was resuspended in 4 mℓ of complete culture medium with 10% (v/v) fresh, unparasitized bovine erythrocytes and distributed equally in four wells of a 24-well culture plate. The plate was incubated in a modular incubator chamber at 37 °C in an atmosphere of 2% O_2, 5% CO_2 and 93% N_2. Medium was changed daily by the replacement of 700 µℓ of medium overlying the erythrocytes in each well.

When the PPE reached about 0,2%, culture plates were transferred in a 5%-CO_2-in-air atmosphere at 37 °C.

Erythrocytes

Blood cells were washed five times by centrifugation (650 x *g*, 10 min, 4 °C) and resuspension in mVYM. After each wash the leucocytes were removed from the interphase of the supernatant and bovine-red-blood-cell (BRBC) suspension. After the last wash, the BRBCs were suspended in mVYM solution at a concentration of 50% (v/v) and stored at 4 °C until use, but not longer than 2 weeks.

Subcultures

Erythrocytes in the culture wells were resuspended and 0,5 mℓ (1:2, v/v) or 0,25 mℓ (1:4, v/v) was transferred into each of two and four new wells, respectively. To each of the wells was added a 10% suspension of uninfected BRBCs in complete medium to make up a final volume of 1 mℓ.

Estimation of parasite growth

Cultures of *Babesia* sp. changed colour from bright red to almost black while they were growing continuously. This is similar to what was described for *B. bovis*-infected cultures (Levy and Ristic 1980). To determine the PPE, culture samples were smeared on microscopic slides, fixed with methanol, Giemsa-stained (10% v/v; 45 min), and 1 000 cells were counted.

RESULTS

Two days after initiation, parasites were detected in smears prepared from cultured material. On day 4, the cultures were split at a ratio of 1:2 (v/v) into a new plate. The original plate was left as safety back-up in the gas mixture, whereas the new plate was transferred into a humidified atmosphere of 5% CO_2 in air. Both approaches were equally good, so that on day 5 the initiation plate was also moved into the regular CO_2 incubator. Starting from day 6, cultures were split daily at a ratio of 1:4 (v/v). Bovine and equine serum-supplemented media supported the initiation and the propagation of the parasites equally well. The average PPE obtained in culture ranged from 2–5%.

When the culture media supplemented with bovine serum did not receive additional purine bases, the PPE declined. After 10 d, the PPE was about 0,4% compared to control cultures with an average PPE of 3–4%. This first became apparent in the lighter colour of the cultures on day 4 after they had changed to the extra purine-deprived medium. However, when the purine-supplemented medium was reapplied, at which stage only a few parasites were detected, the growth rate returned to levels of the control wells.

Microphotographs of cultured *Babesia* sp. are shown in Fig. 1. Most of the culture-derived parasites were piriform and paired (Fig. 1a, 1b). Multiple parasites within a cell were seen occasionally (Fig. 1c). A trophozoite is shown in Fig. 1d.

DISCUSSION

The unclassified *Babesia* sp. originally isolated from an ox (De Waal *et al.* 1990) was adapted to *in vitro* conditions by the use of methods described for the bovine *Babesia* spp., *B. bovis* (Rodriguez, Buening, Green & Carson 1983) and *B. bigemina* (Vega, Buening, Green & Carson 1985). The medium used consisted of HL-1, a medium originally designed for the serum-free cultivation of hybridoma cells. HL-1-based media were previously used for the isolation and cultivation of several *Babesia* species, namely *B. caballi* (Holman, Frerichs, Chieves & Wagner 1993), *B. equi* (Holman, Chieves, Frerichs, Olson & Wagner 1994), a *Babesia* sp. from a North American elk (Holman, Craig, Doan Crider, Petrini, Rhyan & Wagner 1994), and another species from an American woodland caribou (Holman, Petrini, Rhyan & Wagner 1994). Cultures were initiated from stabilates

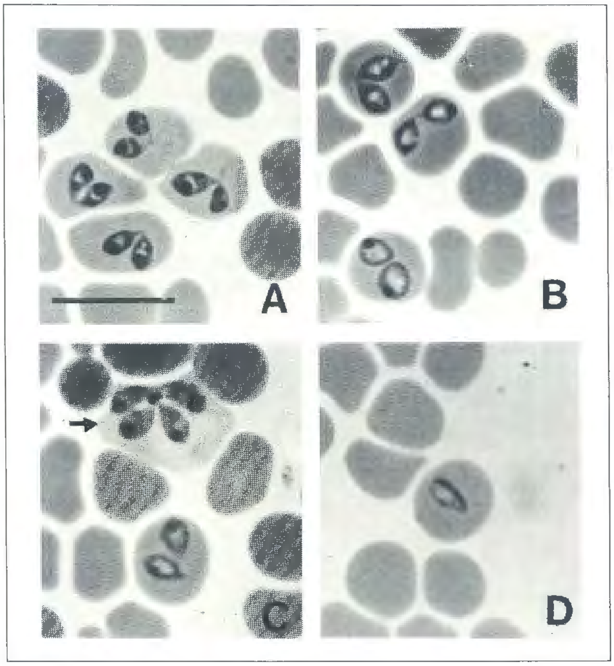

FIG. 1 Giemsa-stained smears of the *Babesia* sp. from an ox cultured in the 23rd passage. A, B. Paired piriform parasites C. Multiple parasites (arrow) within a cell. D. Trophozoite

Bar represents 10 µm

with an initial PPE ((before cryopreservation) as low as 0,002 %. Compared to the use of a splenectomized animal to generate parasites for culture initiation, this new approach saved time and money.

The parasites multiplied promplty and cultures could be subcultured as early as 4 d after initiation, giving rise to continuous cultures, irrespective of whether equine- or bovine-serum-supplemented media were used. Similar results were obtained by Van Niekerk and Zweygarth (manuscript in preparation) for the initiation and propagation of *B. occultans* in medium supplemented with bovine or equine serum. Adaptation of *B. bovis* to equine serum needed a gradual substitution of bovine serum by the former (Yunker, Kuttler & Johnson 1987) whereas Fish, Pipano, Shkap & Frank (1993) were able to change from bovine- to equine-serum-supplemented medium without an adaptation period.

When cultures were fed with bovine-serum-based medium without additional purines, the PPE declined rapidly, and the usual subculture intervals could not be maintained. This process was, however, still reversible after 10 d of cultivation, and parasites resumed normal growth when complete medium was re-supplied. This negative influence on parasite growth was found only with bovine-serum-supplemented media, whereas medium containing horse serum apparently contained enough purine bases to sustain a normal growth. *Babesia* spp. depend on the salvage of preformed purines (Sherman 1984), therefore they readily incorporate hypoxanthine (and other purines) into their nucleic acid (Irvin, Young and Purnell 1978; Irvin and Young 1979; Conrad 1986). In the complete medium used here, the purine concentration obviously became a limiting factor for the growth of *Babesia* sp. since either hypoxanthine, adenosine, guanosine or adenine supplementation reversed the decline in parasite growth. These results were in striking contrast to those of Konrad, Phipps, Canning and Donnelly (1984) who found that medium supplemented with hypoxanthine was even deleterious to *B. divergens* cultures.

Culture-derived parasites will be used to isolate small subunit ribosomal RNA which will be amplified by means of the polymerase chain reaction, cloned and sequenced to clarify the taxonomic position of this *Babesia* species.

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