RESEARCH COMMUNICATION

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

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ABSTRACT


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% O2, 5% CO2 and 93% N2 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% CO2 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
& 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 Martínez phosphate-buffered saline solution (mVYM) (Zweygarth, Just & De Waal 1995). After centrifugation (2000 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% O₂, 5% CO₂ and 95% N₂. 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%-CO₂-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 & Ristic 1980). To determine the PPE, culture samples were smeared on microscopic slides, fixed with methanol, Giemsa-stained (10% v/v; 45 min), and 1000 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% CO₂ in air. Both approaches were equally good, so that on day 5 the initiation plate was also moved into the regular CO₂ 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
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 promptly 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 supplementation 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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