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Continuous in vitro Cultivation of *Babesia ovata*

IKUO IGARASHI[1], AVGAANDORJIIN AVARZED[2], TETSUYA TANAKA[2], NOBORU INOUE[2], MASANARI ITO[2], YOSHITAKA OMATA[2], ATSUSHI SAITO[2] AND NAOYOSHI SUZUKI[1]

[1]*The Research Center for Protozoan Molecular Immunology and* [2]*Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan.*

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ABSTRACT

Basic method for in vitro cultivation of *Babesia ovata* was examined using a method which was developed by Vega et al. for cultivation of *B. bigemina*, a closely related organism. The parasites obtained from an infected SCID mouse were initiated their multiplication within adult bovine RBC in Medium 199 supplemented with 40% adult bovine serum under a low oxygen atmosphere, 5% O_2, 5% CO_2 and 90% N_2. Although no proliferation of *B. ovata* maintained in 5% CO_2 in air was seen during the initial 5 days, the parasites passaged three times under the low oxygen atmosphere were readily cultured in 5% CO_2 in air, as well as under the low oxygen atmosphere. The parasites were propagated in the RBC stored in Vega y Martinez solution at 4°C for up to 2 months. *Babesia ovata*-infected RBC from cultures were successfully cryopreserved in 10% polyvinyl-pyrrolidone in Vega y Martinez solution and used to initiate new culture not only under the low oxygen atmosphere but also in 5% CO_2 in air.

INTRODUCTION

The success of continuous cultivation of *Babesia bovis* with microaerophilous stationary phase system (Levy and Ristic 1980) stimulated the cultivations of other *Babesia* parasites such as *B. divergens* (Vaayrynen and Tuomi 1985), *B. canis* (Molinar et al. 1982), *B. gibsoni* (Onishi et al. 1993), *B. caballi* (Holman et al. 1993) and *B. equi* (Holman et al. 1994), and these success of in vitro cultivation contributed to the morphological and biochemical study, drug sensitivity and supply of antigen or immunogens for the prevention of disease (Kellermann et al. 1985).

Babesia ovata is a relatively large *Babesia* parasite and widespread in Japan. It is not highly pathogenic for cattle, but splenectomized cattle show high parasitemias accompanied by anemia, hemoglobinuria, and in some cases, death (Ishihara and Minami 1977; Minami and Ishihara 1980). Morphologically, the intraerythrocytic forms of *B. ovata* are closely resembled *B. bigemina*. Vega et al. (1985a) reported that *B. bigemina* was cultured continuously under in vitro labo-

ratory conditions, and they also established a cryopreservation method of cultured *B. bigemina* (Vega et al. 1985b). We applied these methods to the cultivation of *B. ovata* by taking advantage of morphological similarity of two parasites. The present study reports establishment of in vitro culture of *B. ovata* and of cryopreservation of the parasite.

MATERIAL AND METHODS

Parasites: *Babesia ovata* (Miyake strain) was provided from the Kyushu Branch of National Institute of Animal Health (Kagoshima, Japan). A frozen stabilate of *B. ovata* was inoculated into, instead of cattle, a splenectomized SCID mouse which was transfused with bovine RBC, according to the method of Tsuji et al. (1992). When parasitemia reached at 2.3%, infected RBC were collected from the SCID mouse by a cardiac puncture with a syringe containing glass beads, and the defibrinated blood was then washed twice with Vega y Martinez (VYM) phosphate-buffered saline solution (Vega et al. 1985a) and used for in vitro cultivation.

Culture media: Three culture media, RPMI 1640, Medium 199 (M 199) and Minimum Essential Medium (MEM), were purchased from Flow Laboratories (ICN Biomedical Inc., CA, USA). The culture media contained $NaHCO_3$ (2.2g/l) or 25mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and pH of all the media was adjusted to 6.70-6.90 and supplemented with bovine serum. Antibiotics were not added to any culture media used in the experiment.

Bovine erythrocytes and sera: Normal venous blood from adult Holstein cows of the University farm was aseptically collected and was immediately defibrinated by shaking with glass beads. The defibrinated blood was centrifuged at 800 *g* for 10 min at 4°C and serum was separated and distributed into sterile 15 ml plastic tubes in 4.0 ml aliquots, and stored at -80°C until use. Remaining RBC were subsequently washed twice in VYM solution at 800 *g* for 10 min at 4°C to remove buffy coat cells, and resuspended in VYM solution to a final 30-50% concentration for storage at 4°C until use. Fetal bovine and calf sera were purchased from ICN Biomedical Japan Co. Ltd. (Osaka, Japan).

Culture conditions: An infected RBC suspension was prepared at 10% concentration (v/v) by mixing one part of packed RBC with 9 parts of serum-supplemented medium. Duplicate RBC suspensions were placed in wells of sterile, disposable 24-well multiple plates (one ml/well) (Corning Laboratory Sciences Company, U. S. A.) and incubated under a low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) or in 5% CO_2 in air. Culture medium was changed daily with fresh medium.

Cryopreservation of parasites: Stock solution of 20% polyvinylpyrrolidone (PVP) was made in VYM solution according to Vega et al. (1985b) and used as a cryoprotectant solution. When parasitemia was higher than 5%, packed infected RBC were mixed with an equal volume of 20% PVP and suspended in 1.5 ml cryotubes. The PVP concentration of the final mixture was 10%. The vials were rapped with tissue papers and frozen at -80°C overnight. The vials were then stored in liquid nitrogen following day.

The frozen vials were taken from liquid nitrogen container and rapidly thawed in a 37°C water bath. The thawed samples were immediately diluted in 10 ml of VYM and centrifuged at 800 *g* at 4°C for 10 min. The resulting pellets were resuspended in fresh culture medium containing 10% normal bovine RBC and one ml of RBC suspension was dispensed in wells of 24-well microplates. The

plates then were incubated under the low oxygen atmosphere or in 5% CO_2 in air. Culture medium was changed daily and the growth of parasite was monitored in Giemsa-stained blood smears by means of light microscopy.

RESULTS

Infected blood was obtained from the splenectomized SCID mouse infected with *B. ovata* at the time of initiation of culture. The defibrinated and washed blood was suspended in a mixture of M 199 supplemented with 40% adult bovine serum containing 10% normal RBC (v/v). One ml of suspension was transferred to a well of two separate 24-well microplates. The plates were incubated in humidified atmosphere of 5% CO_2 in air or under the low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) at 37°C. No growth of parasites was observed in culture in 5% CO_2 in air. In contrast, growth of *B. ovata* was observed in the cultures maintained under the low oxygen atmosphere (Fig. 1). Although parasitemia was very low (less than 0.3%) until day 3, thereafter parasitemia steadily increased on days 4 and 5 (0.6% and 1.2%, respectively). Subculture was made on day 5 by diluting 1:2 with a 10% normal RBC suspension. The second subculture was carried out on day 9 by diluting 1:10 with 10% normal RBC suspension when parasitemia reached at 4.2%. The parasites were then subcultured every 3 to 5 days depending on the growth of parasites, and maintained continuously in vitro for more than 1 year. Parasitemias in excess of 6% were frequently observed. After three passages of parasite under the low oxygen atmosphere, growth of parasite was observed in 5% CO_2 in air, as well as under the low oxygen atmosphere (Fig. 2).

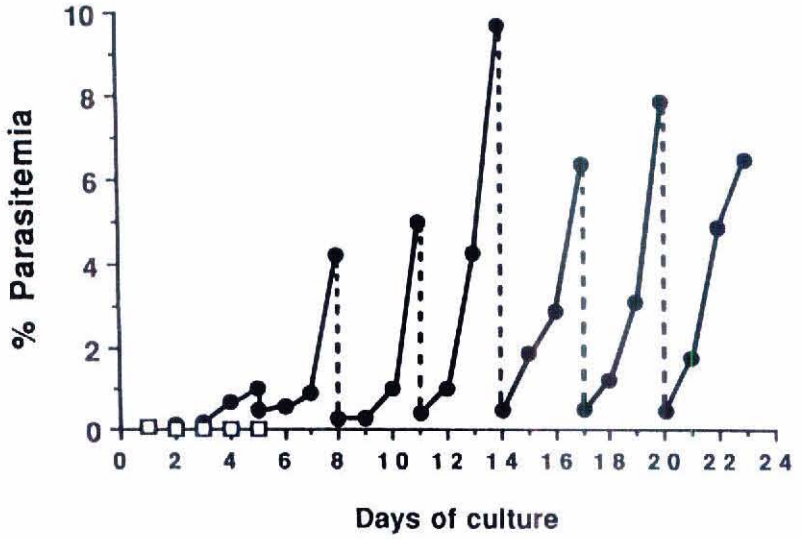

Fig. 1. Growth of *B. ovata* cultured from the blood of an infected SCID mouse in different atmospheres. Days at subcultures are indicated with dotted lines. Symboles; low oxygen, 5% O_2, 5% CO_2 and 90% N_2 (●) and 5% CO_2 in air (□).

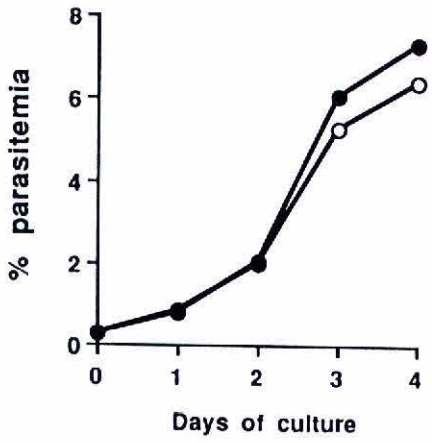

Fig. 2. Growth of *B. ovata* in different atmospheres after three passages. Symboles; low oxygen (○) and 5% CO_2 in air (●).

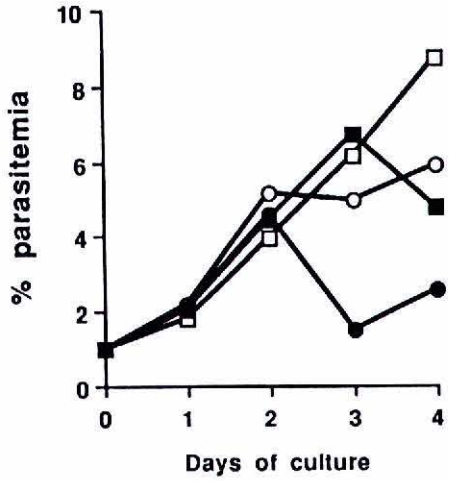

Fig. 3. Growth of *B. ovata* using various culture media. Symboles; $NaHCO_3$-buffered M 199 (□), HEPES-bufferedM 199 (■), $NaHCO_3$-buffered MEM (○) and $NaHCO_3$-buffered RPMI 1640 (●).

Three culture media, M 199, RPMI 1640 and MEM buffered with $NaHCO_3$, and one medium, M 199 buffered with HEPES, were examined on the growth of parasites. All the media contained 40% adult bovine serum. No significant difference in parasitemia was found among the 4 media on day 2. However, M 199 buffered with $NaHCO_3$ supported higher parasitemia than other three media on day 4 (Fig. 3). $NaHCO_3$ showed superior effect to support the parasite growth than HEPES when it was added to M 199. Therefore M 199 buffered with $NaHCO_3$ was selected for further experiments.

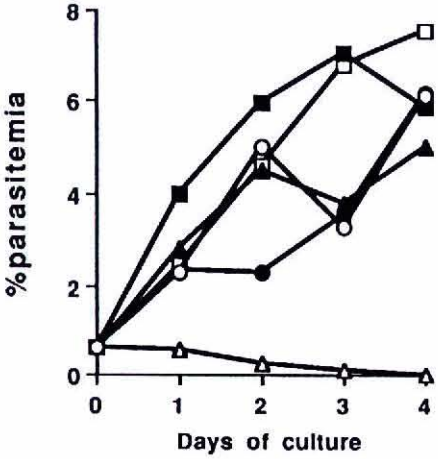

Fig. 4. Growth of *B. ovata* using adult bovine serum at different concentrations with M 199. Symboles; 0% (△), 20% (●), 40% (□), 60% (■), 80% (▲) and 100% (○).

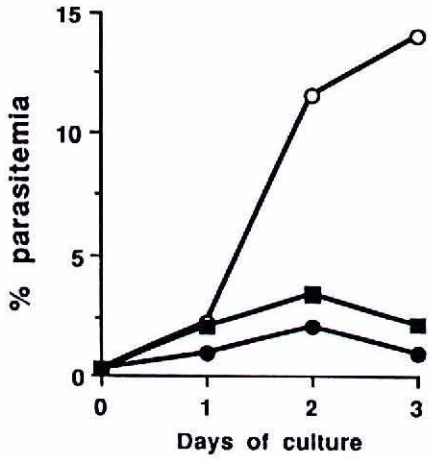

Fig. 5. Growth of *B. ovata* using different sera with M 199. Symboles; adult bovine serum (○), calf serum (●) and fetal bovine serum (■).

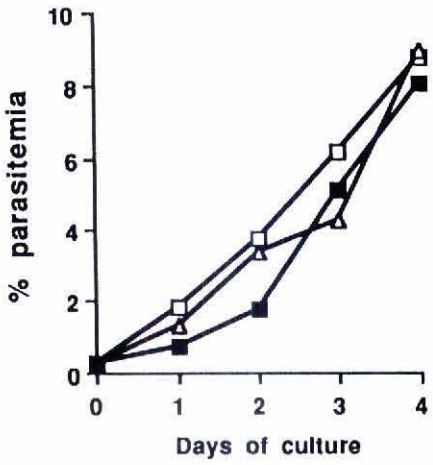

Fig. 6. Growth of *B. ovata* in normal adult bovine RBC stored at 4 °C for different periods. Symboles; freshly collected (□), stored for 37 days (△) and stored for 57 days (■).

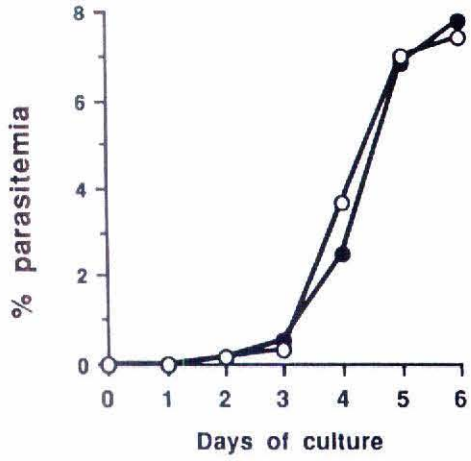

Fig. 7. Growth of *B. ovata* in different atmospheres after cryopresrvation. Symboles; low oxygen atmosphere (○) and 5% CO_2 in air (●).

After $NaHCO_3$-buffered M 199 was selected in the previous experiment, the effect of different concentrations of adult bovine serum on the growth of parasites was examined. Although 20% or higher concentrations seemed to support the growth of parasites, the highest parasitemia was observed at the 40% of serum concentration (Fig. 4). However, supplement with 40% of fetal bovine serum or calf serum did not support the growth of parasite (Fig. 5). Heat-inactivation of serum (at 56°C for 30 min) did not affect the growth of parasites (data not shown).

The effect of storage of adult bovine RBC on the growth of parasites was examined for practical consideration. Normal RBC were collected on day of experiment or collected from the same donor and had been stored for 37 and 57 days in VYM solution at 4°C, respectively. No difference of the parasite growth was seen in RBC stored for different periods of time (Fig. 6). As the result of this finding, normal RBC were stored for up to 2 months in VYM solution at 4°C.

Cultured parasites from the sixth passage in the RBC were cryopreserved using 20% PVP in VYM buffer as a cryoprotectant. Cultures were initiated again from the cryopreserved parasites propagated under the low oxygen atmosphere. Low numbers of *B. ovata* within RBC were first observed on the day 2 after the initiation of culture. On days 4 and 6, parasitemias of culture were 0. 3% and 3.6%, respectively. On day 7, at the time when the parasitemia was 4.9%, first subculture was made. The parasitemia in the subculture was reached at 7.7% three days after the initial subcultivation, thereafter successful subcultures were made every 3-5 days. In a separate experiment, the effect of culture atmosphere on the cryopreserved *B. ovata* was examined by initiating cultures again from the cryopreserved materials either under the low oxygen atmosphere or in 5% CO_2 in air. The atmosphere of 5% CO_2 in air, as well as the low oxygen atmosphere, supported the growth of parasite (Fig. 7).

DISCUSSION

The present study demonstrated that *B. ovata* could be cultivated continuously in vitro using a method which was developed for in vitro cultivation of *B. bigemina*, a closely related organism. A low oxygen atmosphere (5% O_2, 5% CO_2 and 90% N_2) was necessary to initiate growth of *B. ovata* in bovine RBC. The reduced oxygen requirement for in vitro culture is also reported in *B. bigemina* (Vega et al. 1985a), *B. caballi* (Holman et al. 1993) and *B. equi* (Holman et al. 1994). These results suggest that oxygen gas tension seems to be a key factor for initiation of in vitro culture of *Babesia* parasites. After three passages, however, *B. ovata* could be maintained in bovine RBC culture under 5% CO_2 in air (high-O_2) as observed in *Plasmodium falciparum* (Waki et al. 1984) and *B. bigemina* (Vega et al. 1985b). The oxygen requirement of *B. bovis* was varied according to initial parasitemia of subculture (Rodriguez et al. 1982). *Babesia bovis* grew readily in cultures in 5% CO_2 in air when an initial parasitemia was 0.5% or above, but the parasites did not when the initial parasitemia was 0.1% or less. If this similar effect occurred with *B. ovata*, threshold of the growth of *B. ovata* in 5% CO_2 in air might be lower than of *B. bovis*.

Serum supplement to medium is essential to in vitro growth of *B. ovata*. Cultures without serum did not support even limited parasite multiplication, but cultures containing greater than 20% serum supported the growth. Cultures with 40% of serum concentration showed the highest multiplication. Furthermore, effects of fetal bovine and calf sera on the the growth of parasites were compared with those of adult bovine serum, since young animals are known to be more resistant to *Babesia* infection than adults (Levy and Ristic 1981). Commercially obtained fetal bovine and calf sera did not support the growth of *B. ovata*. One possible explanation may relate to the presence of an inhibitory factor(s) to parasite growth in young animals as suggested in *B. bovis* (Levy et al. 1982), or lacking a supporting component(s) for growth of *B. ovata*. Further study of effect of RBC and leukocytes from young animals will need to determine the role of blood components in the phenomenon of age resistance.

The storage of RBC for longer period is very important for practical consideration of this culture method. The present study demonstrated that storage of RBC in VYM buffer for up to 2 months at 4°C did not affect parasite growth and during the period severe hemolysis was not observed. This finding is contrast to the cultivation of *B. bovis* and *B. bigemina*, where that RBC can be stored at most for one week only (Vega et al. 1985a; Levy and Ristic. 1980). De Verdier et al. (1981) reported the favorable effect of citrate-phosphate-dextrose supplemented with adenine-guasonine on the stability of human blood stored at 4°C. High concentration of glucose in VYM solution supplies energy source for RBC, and adenine and guasonine enhance ATP and 2,3-DPG production, and ATP is closely linked to preservation of biconcave shape of RBC. An addition of adenine to human blood preservation media extended the storage period of RBC from 21 to 28 days or 35 days (Sasakawa 1983). These favorable effects may occur with respect to the storage of bovine RBC for longer periods in the case of *B. ovata* cultivation.

The reestablishment of in vitro culture, using frozen *Babesia* parasites, would eliminate the need for maintaining infected animals. The successful cryopreservation of *B. bovis* for initiation of in vitro cultures was reported by Palmer et al. (1982) using 10% PVP in Pack's saline G solution supplemented with extra glucose. However, this cryoprotectant solution did not work with *B. bigemina*.

Vega et al. (1985b) developed a modified procedure for cryopreservation of *B. bigemina* with 10% PVP in VYM solution instead of Pack's saline G. Application of this procedure to *B. ovata* was successful in the initiation of culture of cryopreserved parasites and animal inoculation (data not shown). Growth of *B. ovata* was observed in cultures under a low oxygen atmosphere (5% CO_2, 5% O_2, 90% N_2) as observed in *B. bigemina* (Vega et al. 1985b). Furthermore, *B. ovata* could be also cultured in 5% CO_2 in air. This result may indicate that high infectivity of cryopreserved *B. ovata* was retained during cryopreservation of parasites, since relatively high oxygen atmosphere of 10% O_2, 5% CO_2, and 85% N_2 could support the initiation of growth of *B. bigemina*, if a high ratio of infectivity was used (Vega et al. 1985b). These results suggest that 10% PVP in VYM solution is highly suitable to cryopreserve *B. ovata*.

The present study suggests that *B. ovata* can be cultured readily using a conventional CO_2 incubator after establishment of in vitro culture or cryopreservation of parasites from cultured materials. By establishment of in vitro cultivation, animal infection for maintaining parasites and the risk of spread to susceptible animals could be avoided. In addition, in vitro cultivation can be used to produce specific antigen for serology, and to study the life cycle and biochemistry of parasites and to develop effective and safe vaccines.

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