Continuous in vitro Culture of Erythrocytic Stages of Babesia gibsoni and Virulence of the Cultivated Parasite

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ABSTRACT. Babesia gibsoni infected erythrocytes were collected from the blood of an experimentally infected dog. The parasite isolated could be continuously cultivated in vitro, with an average parasitemia of 18.2 ± 2.4% on day 3 of culture, in RPMI-1640 medium supplemented with 7.5% normal dog serum in a humidified atmosphere containing 5% CO2 at 37°C. The parasites in the original culture were morphologically similar to those found in the peripheral blood of dogs, however, on the 4th generation of subculture, the large oval parasites, erythrocytes including many parasites and extracellular parasites were frequently observed. The B. gibsoni isolate was injected to the dog to test its infectivity after maintained in vitro for 738 days at the 214th subculture. The cultivated parasite did not cause a severe clinical sign in the dog.

KEY WORDS: Babesia gibsoni, canine, in vitro culture, infectivity, morphology.

Babesia gibsoni is an erythrocytic parasite, which is transmitted to dogs by ticks and causes hemolytic anemia, hemoglobinuria and marked splenomegaly in dogs, and the parasites, erythrocytes including many parasites and extracellular parasites were frequently observed. The B. gibsoni isolate was injected to the dog to test its infectivity after maintained in vitro for 738 days at the 214th subculture. The cultivated parasite did not cause a severe clinical sign in the dog.

MATERIALS AND METHODS

Three beagles, 1 to 2 years old were used in the experiments. The dogs were confirmed free of natural B. gibsoni infection by the indirect fluorescent antibody test and microscopic examination of Giemsa-stained blood smear before use. They received a standard amount of food daily and drinking water ad libitum. Two of the dogs were used as the donors of normal erythrocytes and serum and the other one was used for the examination of infectivity and virulence of cultured B. gibsoni.

The culture medium was RPMI-1640 (GIBCO) supplemented with 25 mM HEPES (N-[2-hydroxyethyl]piperazine- N’-[2-ethanesulfonic acid]), 2 mM L-glutamine, 1 mM pyruvic acid, 24 mM NaHCO3, penicillin G at 100 units/ml and, streptomycin at 100 µg/ml. Blood to supply normal dog erythrocytes was collected from the donor dogs with a heparinized syringe. The blood was added to an equal volume of culture medium and the normal dog erythrocytes suspension was stored at 4°C until use. Normal serum was prepared from blood collected from the donor dogs and stored at –20°C until use.

A B. gibsoni (B. gibsoni Oita isolate) was isolated from an infected dog in Oita Prefecture, Japan, in 1978 and is maintained in our laboratory by passage through both splenectomized and normal dogs. The morphology and virulence of this isolate was reported previously [10–12, 14]. Infected blood samples for culture were collected from a dog inoculated with B. gibsoni Oita isolate, with a heparinized syringe and showed 2.0% parasitemia and 39% packed cell volume (PCV). The blood cells were washed three times by centrifugation at 2,000 rpm for 10 min at 4°C and suspended in phosphate buffer solution (PBS). To determine the optimum serum concentration, the erythrocytes were suspended to become a final PCV of 10% in the culture medium supplemented with 10, 20, 30, 40 and 50% of dog serum respectively, and 2 ml of the suspension each was introduced 2 into 12 wells of a culture plate. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 6 days. Every 24 hr, 1 ml of the culture supernatant was obtained by using RPMI-1640 supplemented with 20% dog serum in a humidified atmosphere containing 5% CO2 at 37°C and exchanging the medium at 24-hr interval. The parasites were maintained at least for 28 days by exchanging one third of erythrocytes at every seven days after cultivation. However, long term in vitro cultivation of B. gibsoni with a high level of parasitemia has not been successfully achieved until now.

We cultivated a B. gibsoni Oita isolate for 814 days with a high level of parasitemia and observed some changes in the morphology, infectivity and virulence of the isolate by in vitro culture.
The supernatant was removed without disturbing the sedimented erythrocytes and replaced with an equal volume of each fresh medium. Small samples were collected from each well daily to monitored percentage parasitemia by microscopic examination of Giemsa-stained blood smears. Parasitemia was determined by counting the infected erythrocytes in 1,000 red blood cells (RBC) on a slide. The effect of different serum concentration on in vitro growth of the parasite was examined on the 75th and 100th subcultures again. The significance of differences was examined using Student’s t-test.

Subcultures were usually prepared every 3 days, but the interval varied from 3 to 12 days until the 28th subcultures because of unstable multiplication of the parasite. After 1 ml of the supernatant medium was removed, 330 µl of the infected erythrocytes suspension was transferred to a well containing 1,330 µl of fresh medium and 330 µl of normal dog erythrocytes suspension. The cultures were then incubated in an atmosphere containing 5% CO2 at 37°C. One ml of the supernatant medium was replaced daily by fresh medium and parasitemia was monitored. We could, successfully cultivate the B. gibsoni Oita isolate for 814 days at the 239th subculture.

The cultivated B. gibsoni Oita isolate was tested for infectivity and virulence by injecting 10⁹ infected erythrocytes included in 2.5 ml of the sample intravenously into a dog. The sample for inoculation was collected from the suspension on day 3 of cultivation at the 214th subculture. Blood cells in the sample suspended in PBS were washed three times by centrifugation at 2,000 rpm for 10 min at 4°C. The sample showed 20.3% parasitemia and 200 × 10⁴/µl RBC. Parasitemia, PCV and rectal temperature were monitored daily for 50 days after injection.

RESULTS

Parasitemia increased, in general, with time after cultivation, but its increase differed in different concentrations of serum in culture medium (Fig. 1). Parasitemia increased significantly (p<0.05) higher at 20% to 40% serum concentration than at 10% and 50%. Parasitemia fluctuated in each subculture as shown in Fig. 2. In the original culture, parasites grew well and parasitemia reached 21.0%. After that, parasitemia was abruptly reduced to less than 0.1% in the 8-10th and 20–21st subculture. However, it recovered from the 23rd subculture, and parasites grew stably and parasitemia always showed 3.6 ± 1.3% on 3 day of cultivation. On the 75th subculture, two different serum concentrations, 10% and 20%, were compared as to their effect on in vitro growth of parasites again. Parasitemia was insignificantly different between 20% (9.4 ± 3.5% parasitemia, n=5) and 10% serum concentration (9.2 ± 1.9% parasitemia, n=5) on day 3 of cultivation. The serum concentration used was 10% after the 75th subculture. Furthermore, on the 100th subculture, 10% and 7.5% serum concentrations were compared, and parasitemia was insignificantly different between 10% and 7.5% serum concentration on day 3 (data not shown). The serum concentration used was 7.5% after the 100th subcultures. After many generations of subcultivation, parasitemia gradually increased.

![Fig. 1. Effect of different serum concentrations on in vitro growth of B. gibsoni. 10% (-□-), 20% (-▲-), 30% (-■-), 40% (-●-), 50% (-○-). Vertical bars indicates mean ± standard deviation of two samples. An asterisk (*) shows statistically significant difference from other values by Student’s t-test at p<0.05. Parasitemia increased significantly (p<0.05) higher at 20% to 40% serum concentration than at 10% and 50%.

![Fig. 2. Change of parasitemia in the 1st-36th subcultures.](image-url)
The average parasitemia of the 190–199th subcultures was 18.2 ± 2.4% on day 3 of cultivation, however, parasitemia did not increase more than 18.2 ± 2.4%, even if subcultivation was continued more than 230 times (Fig. 3).

*B. gibsoni* organisms in the original culture were morphologically similar to those in the peripheral blood, which assumed the oval and dot-forms (Fig. 4-1). The oval parasite was 1.87 ± 0.40 µm × 1.60 ± 0.28 µm in mean size (n=30) on day 4 of the original culture. On the 4th subculture, other forms of parasites were observed, such as, the large oval parasites, more than 3.0 µm in length, inside and outside the host cell, the clusters of extra cellular parasites which indicated the rupture of erythrocytes parasitized by the petaloid-shaped parasites, and erythrocytes including multiple parasites (Fig. 4-2). On day 3 of the 5th subculture with 2.8% parasitemia, the proportion of erythrocytes including different numbers of parasites was as follows: a single parasite, 83%; 2 parasites, 10%; 4 parasites, 3%, and more than 8 parasites, 4%. In the 8th-10th and 20th–21st subcultures when the maximum parasitemia was less than 0.1%, no parasites were observed outside host cells and only a single parasite was observed in the erythrocytes. However, after the 23rd generation of subculture, the large oval and petaloid-formed parasites appeared again in the erythrocytes and the parasites were also present outside the cells. On day 3 of the 30th subculture with a parasitemia of 5.8%, the proportion of erythrocytes parasitized with different numbers of parasites was as follows: with a single parasite, 70%; 2 parasites, 19%; 4 parasites, 8% and more than 8 parasites, 3%.

The cultivated *B. gibsoni* Oita isolate at the 214th subculture was tested for infectivity and virulence by injecting 10⁹ infected erythrocytes included in 2.5 ml of suspension intravenously into the dog. The change of parasitemia and PCV with time after inoculation is shown in Fig. 5. The parasite was observed in the peripheral blood on days 3–6 after injection, when parasitemia was less than 0.01%. However, parasitemia increased again to 4.4% on day 34. PCV transiently decreased. The body temperature did not exceed...
40°C during the experimental period. At the peak of parasitemia, the oval parasites were $2.18 \pm 0.38 \mu m \times 1.90 \pm 0.30 \mu m$ (n=30) in mean size, and the erythrocytes including a single and 2 parasites were 95% and 5%, respectively. Neither multiple infections of erythrocytes with more than 4 parasites nor the parasites outside the erythrocytes were observed.

DISCUSSION

Several Babesia species have been successfully cultured in vitro [2,15]. Levy and Ristic reported the cultivation method of Babesia bovis based on the microaerophilous stationally phase system [7]. The in vitro cultivation method of B. gibsoni reported by Murase et al. [8] was also a modification of the above system, in which parasites were cultured in a microtiterplate to supply efficient oxygen. They cultured the parasites in α-medium supplemented with 40% normal dog serum in a humidified atmosphere containing 5% CO2 at 37°C for 15 days and the maximum parasitemia was 4.0% on day 8 of incubation. Onishi et al. [13] cultured the parasites in RPMI-1640 medium plus 20% normal dog serum in a humidified atmosphere containing 5% CO2 at 37°C for 28 days with the maximum parasitemia of 9.5% on day 7 of incubation. But the number of parasites decreased gradually 1 week after incubation and the parasites did not grow for 4 weeks after incubation in spite of supplying fresh medium and red blood cells. Zweygarth and Lopes-Rebollar [17] cultured B. gibsoni from Sri Lanka and Bangladesh stock in HL-1 medium supplemented with 40% normal dog serum with the maximum parasitemia of 1.3 to 5.6%. They reported that a concentration of 40% dog serum was crucial during the early stage of culture, which could be lowered to 20% after the initiation phase, and that HL-1 medium supported the growth of parasites, whereas RPMI-1640 did not. However, we continuously cultivated B. gibsoni using RPMI-1640, although fresh medium and red blood cells were continuously supplied when the parasites came not to grow. After then, the parasites began to increase in number again. In our experiments, initially RPMI-1640 medium supplemented with 20% dog serum was used but the serum concentration could be reduced to 7.5% after the 100th subculture. The average parasitemia was $18.2 \pm 2.4\%$ on day 3 of cultivation. As our results and other workers’ reports show the optimal serum concentration varied from 20 to 40% in the different isolates used at the early stage, and the optimal culture medium was also different in different isolates used, such as α-medium, RPMI-1640 and HL-1 medium. Accordingly, the optimal serum concentration and kind of culture medium need to be examined first. However, in order to maintain the culture for a long time, it may be more necessary to supply fresh medium and erythrocytes continuously, even when no parasites are detected by microscopy, because survival parasites may start to grow again when adapted to the culture medium condition.

Avarez et al. [1] reported that a low oxygen atmosphere is necessary for the initiation of B. caballi cultures, but the level of parasitemia remained low (2–4%) in in vitro culture with a low oxygen atmosphere. They attempted in vain to improve the parasitemia by using different culture media, serum concentrations, or vessel volumes, but the parasitemia became doubled or even greater (8–10%) when parasites were transferred to a 5% CO2 normal atmosphere. Zweygarth and Lopes-Rebollar [17] reported that B. gibsoni cultures required an O2-reduced atmosphere for culture initiation from blood with a low parasitemia (<0.01%); but when blood with a higher parasitemia (2.6%) was used, culture could be initiated out in a normal atmosphere with 5% CO2, and suggested that actively dividing parasites required normal oxygen levels. Besides, Murase et al. [9] reported that parasitemia reached peak levels of $34.1 \pm 15.8\%$ at day 8 of immature-erythrocyte culture, whereas the highest parasitemia attained in mature-cell culture was only $3.6 \pm 2.2\%$ at day 5. Young erythrocytes were the factor to enhance the growth of parasite. Our original culture started from blood with 2.1% parasitemia in normal oxygen level, namely an atmosphere of 5% CO2 in air, and the highest parasitemia was $3.6 \pm 1.3\%$ at day 3 in the 30–39th subcultures. However, parasitemia reached peak levels of $18.2 \pm 2.4\%$ at day 3 of cultivation in the 190–199th subcultures even though mature erythrocytes were used in an same oxygen level.

B. gibsoni organisms in the original culture were morphologically similar to those in the peripheral erythrocytes of the dog experimentally infected with B. gibsoni Oita isolate [10]. The majority of parasites are small oval in shape and occur singly or in pairs in the erythrocytes. On the 4th generations of subculture, the large oval shaped parasites appeared inside and outside the erythrocyte, and several clusters of parasites and the erythrocytes including a lot of parasites were frequently observed. Fukumoto et al. [5] documented that B. gibsoni parasites grown in canine erythrocyte -substituted severe combined immune deficiency (Ca-RBC-SCID) mice showed marked morphological changes, that is, significantly larger parasites than those found in infected dogs and 4, 8, 16 or 32 parasites included in a single erythrocyte were observed. Morphologically, the B. gibsoni parasites in Ca-RBC-SCID mice were similar to those in culture, except for the extra cellular parasites.
Onishi et al. [13] reported that the splenectomized dog intravenously inoculated with $6 \times 10^6$ of B. gibsoni in vitro cultivated for 28 days developed severe clinical signs, which were very similar to those experimentally induced by injection of a large number of parasites. Fukumoto et al. [5] also documented that a dog infected with B. gibsoni (0.5 ml of packed RBCs) propagated in Ca-RBC-SCID mice developed 10% parasitemia and typical clinical symptoms, and further that the parasites restored their original morphology when inoculated in dogs. We described in the previous report that 2 beagles intravenously inoculated with $1 \times 10^7$ B. gibsoni Oita isolate infected erythrocytes developed a severe clinical disease with a parasitemia of more than 20%, PCV of less than 10% and body temperature of more than 40°C [14]. Furthermore, we reported that 5 dogs inoculated with $5 \times 10^7$ B. gibsoni Oita isolate infected erythrocytes showed parasitemia of 10–30% and PCV of 8–15% [11, 12]. However, in the present study, the parasites cultivated for 738 days showed only very weak pathogenicity to the dog. The cultivated parasites were larger in size than those found in natural and experimental infected dogs, as described by other workers [4, 10]. However, no morphological change was observed in the parasite, except for the size of parasites. It is not clear whether any the relation exists between the size and virulence of parasites. Our results suggest that attenuation of the parasite isolate will be possible by the present long-term cultivation method.

REFERENCES