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Short communication

The efficacy of artemisinin, artemether, and lumefantrine against *Babesia gibsoni* in vitro

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ABSTRACT

Artemisinin has many derivatives, and it is effective against *Plasmodium* spp. However, only a limited number of reports have confirmed the efficacy of artemisinin derivatives against *Babesia* spp. In this study, whether artemisinin and artemether could inhibit the growth of *Babesia gibsoni* was evaluated in vitro. In addition, the interaction between artemether and lumefantrine was evaluated. These drugs inhibited the growth of *B. gibsoni*, but artemisinin and artemether showed lower sensitivity against atovaquone-resistant *B. gibsoni* than against wild-type *B. gibsoni*. The interaction between artemether and lumefantrine showed synergism against *B. gibsoni*. Although further study is needed, the combination of artemisinin derivatives could be useful for babesiosis.

Babesiosis is a parasitic disease caused by intraerythrocytic protozoa of the genus *Babesia* and transmitted by ticks. The disease is recognized to be of veterinary importance in cattle, horses, and dogs and is highlighted as an emerging zoonosis in humans. The clinical signs of babesiosis are similar to those of complicated malarial infections including hemolytic anemia, fever, choloplania, hemoglobinuria, and an enlarged spleen [1]. *Babesia gibsoni* infection is recognized frequently in dogs and has been a serious clinical problem worldwide. Some treatment strategies including diminazene aceturate, antibiotic combination therapies, and atovaquone (ATV) have been reported against *B. gibsoni*. Among them, ATV is expected to be used further because it is effective and has less of a side effect. Nevertheless, the disadvantage of this drug is the emergence of drug-resistant variants with a single-nucleotide polymorphism, M121I, in the cytochrome *b* gene which is presumed to be an ATV binding site [2–4]. Additionally, parasites with M121I have been reported in the field in Japan [5]. In order to solve the problems with ATV-resistant *B. gibsoni*, another drug should be examined.

Artemisinin (ART) is extracted from *Artemisia annua* (qinghao), which is a Chinese annual herb. This drug is a sesquiterpene lactone peroxide, and many derivatives are made from this drug by changing the C10 side chain. These derivatives reliably reduce initial malaria parasitemia and are effective against multidrug-resistant malaria. ART can act fast to depolarize the mitochondrial membrane in malaria parasites. It has also been reported that antimalarial activity results from Fe^{2+}-dependent endoperoxide cleavage and that this cleavage forms highly reactive free radicals that alkylate molecules within parasites, eventually leading to their deaths. However, ART and its derivatives have short half-lives, and effective levels in plasma are sustained only for relatively brief periods [6]. In order to better solve these problems, artemether (AT), a water-soluble derivative, is used as a combination therapy with lumefantrine (LMF) [7]. LMF is a fluorine derivative synthesized in the 1970s by the Academy of Military Medical Sciences, Beijing, and has marked blood schizontocidal activity against a wide range of plasmodia. This drug is also an effective antimalarial with a high cure rate, but it acts more slowly. In addition, it is reported that a tablet formula that combines AT and LMF, Riamet®, is available for treating human malaria [8]. Although only a limited number of reports have confirmed the efficacy of ART derivatives against *Babesia* spp., the efficacies of ART derivatives against *Babesia* spp. including the in vitro activity of artesunate against equine *Babesia* spp. [9] and that of artesunate and dihydroartemisinin against *B. gibsoni* [10], have been reported. To our knowledge, there are no reports about the efficacy of ART, AT, and LMF, and the combination of AT and LMF against *B. gibsoni*.

In the present study, we evaluated the efficacy of ART, AT, and LMF and the interaction of AT and LMF against *B. gibsoni* with/without M121I in vitro. The parasites were isolated from a naturally infected Tosa dog in Aomori Prefecture, Japan, in 2004, and they were maintained in vitro culture at our laboratory as WT *B. gibsoni* [2].

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ATV-resistant *B. gibsoni* was developed by exposure to ATV for 144 h [4]. ATV-resistant *B. gibsoni* demonstrated low sensitivity against ATV and cyt *b* of those had M121I. Cultures of these parasites were carried out as reported previously. In brief, 200 μL of packed infected red blood cells was dispensed into 1800 μL of culture medium to obtain a 10% packed cell volume (PCV) in each well of a 12-well plate. Each parasite was incubated at 37 °C in a humidified atmosphere containing 5% CO_2 [2].

The sensitivities of WT and ATV-resistant *B. gibsoni* against ART, AT and LMF were examined using the same technique as reported previously [10]. These drugs were obtained from Sigma-Aldrich (Tokyo, Japan), and stock solutions were prepared with dimethyl sulfoxide (DMSO). ART and AT stock solutions were diluted with a culture medium to yield final concentrations of 1, 3, 10, 30, and 100 μM. The final ART concentration for ATV-resistant *B. gibsoni* was 3–300 μM. LMF stock solution was diluted to 0.001, 0.01, 0.1, 1.0 and 10 μM. Two hundred microliters of each *B. gibsoni* culture suspension was dispensed per well in 96-well plates in triplicate for each drug concentration. Identical cultures containing only DMSO and without each drug were prepared to be used as controls. The final concentration of DMSO was adjusted to 0.1%. These plates were incubated at 5% CO_2 and 37 °C for 6 days. Every day, 80 μL of the medium was replaced with fresh medium, according to the concentration of each drug. The growth inhibition rate of the parasite was calculated by counting parasitized erythrocytes from each of the wells containing drugs and those in the control wells without the drug every 2 days from blood smear [10]. The half-maximal inhibition concentration (IC_{50}) of each drug was determined after 6 days of incubation [10]. Statistical analysis was done using Student's *t* test.

Subsequently, the interactions of AT and LMF against WT and ATV-resistant *B. gibsoni* were examined. In vitro drug interactions were assessed using a modified fixed-ratio isobologram method [11]. After the sensitivity tests, AT and LMF were six-point two-fold diluted, and the IC_{50} was reduced to the midpoint of a dilution series. That is, AT was diluted to 100, 30, 10, 3, 1, and 0 μM and 10, 1, 0.1, 0.01, 0.001, and 0 μM of LMF against both types of *B. gibsoni*. Dilutions of each of the two drugs were prepared in fixed-ratio solutions at ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of AT and LMF, which were serially diluted five times in two-fold dilutions [11]. The final concentration of DMSO

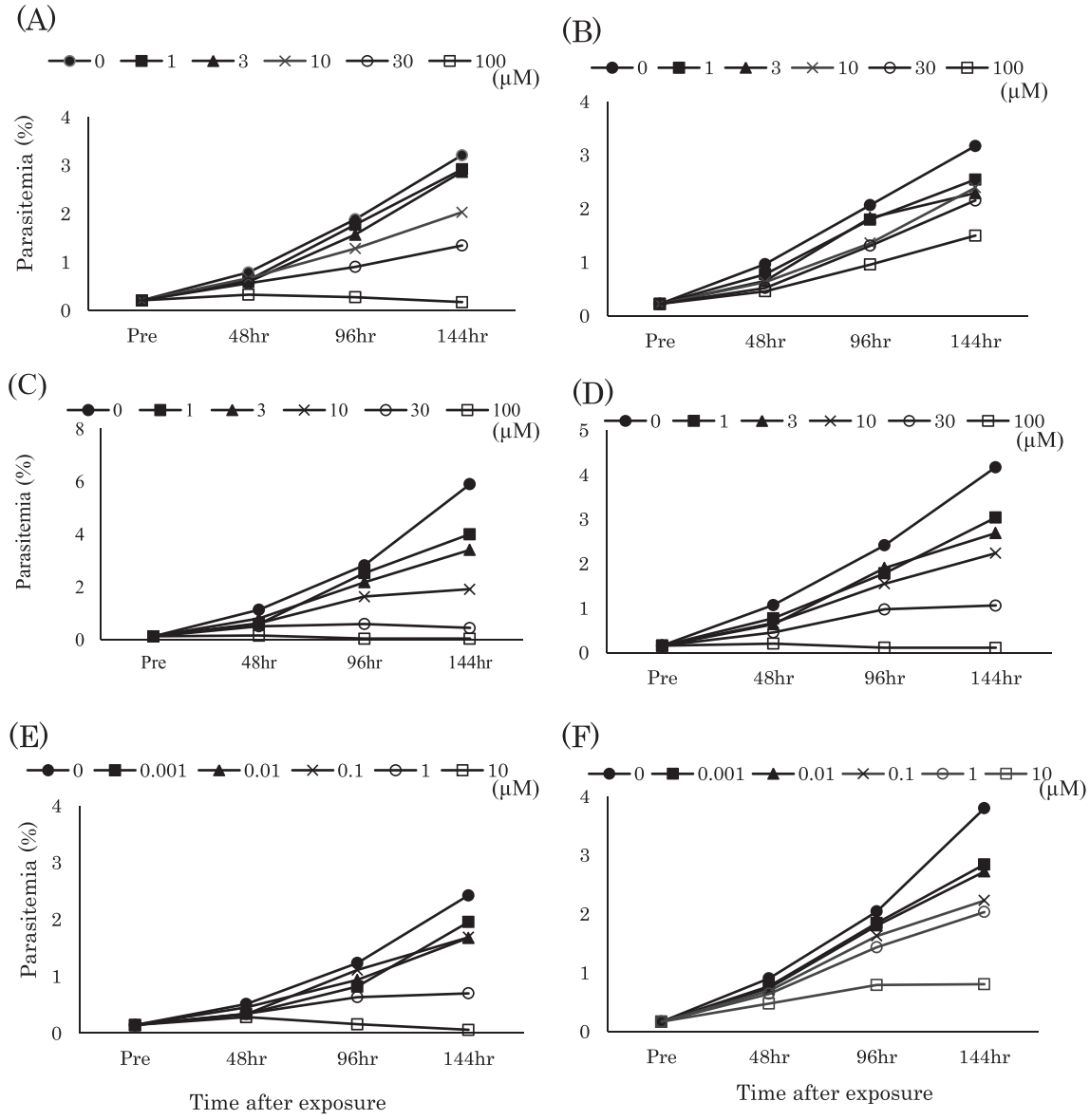

Fig. 1. Growth curves of *B. gibsoni* in in vitro culture treated with different concentrations of ART, AT, and LMF. Data are representative of one experiment of three separate experiments. (A) The sensitivity of ART against WT *B. gibsoni*, (B) the sensitivity of ART against ATV-resistant *B. gibsoni*, (C) the sensitivity of AT against WT *B. gibsoni*, (D) the sensitivity of AT against ATV-resistant *B. gibsoni*, (E) the sensitivity of LMF against WT *B. gibsoni* and (F) the sensitivity of LMF against ATV-resistant *B. gibsoni*.