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Abstract of Dissertation

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(ピロプラズマ症の化学療法と疫学に関する研究)

Abstract

Bovine babesiosis and equine piroplasmosis cause huge economically loss on cattle farming and horse breeding. Besides, zoonotic *Babesia* parasites are frequently identified in humans, rodents, and ticks from the Asian countries. However, prevention and tick control are usually unsuccessfully. Current available drugs have been note side effects on animals, and sometimes drug resistance parasites are reported as well. Hence, antibabesial and antitheilerial drug is desperately needed. Moreover, prevention of zoonotic parasites should be establish in endemic area, but geographical distribution of parasites and their tick vectors still incomplete in most of developing countries.

In the present studies, I revealed that inhibitory effects of methylene blue, dipyridamole, and clofazimine on *in vitro* growths of bovine *Babesia* and *equine* piroplasma parasites and *in vivo* growth of *Babesia microti* in mice were evaluated. The epidemiological study was investigated to detect zoonotic *Babesia* parasites including *B. microti* and *B. venatorum* in ticks from Mongolia, and then genetic characterization was analyzed using on *18S rRNA* for both of parasites, *cox1* and *tufA* genes only for *B. microti*.

In the study on chemotherapy of piroplasmosis, methylene blue, the first fully synthetic drug, has been widely used in medical treatments. A few decades ago, this drug was used as an antimalarial agent. In the present study, methylene blue significantly inhibited the growth of *B. bovis*, *B. caballi*, and *Theileria equi* at a 0.1 μM, while *B. bigemina* was significantly inhibited at 0.01 μM, on day 3 of cultivation. The half maximal inhibitory concentrations (IC_{50}) of methylene blue against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were calculated as 0.83±0.02, 0.68±0.09, 0.54±0.14, and 0.49±0.06 μM, respectively. The subsequent viability assays, in which drug-free media were used for the cultivation, showed that there were no growths of *B. bovis* or *B. bigemina* that had been previously treated with 10 μM methylene blue. Similarly, *B. caballi* and *T. equi* that had been previously treated with 1 μM methylene blue failed to grow in the viability tests. As for the *in vivo* inhibition assay, the high dose of methylene blue showed a low inhibitory effect on the *in vivo* growth of *B. microti*

at 50 mg/kg body weight treatment groups as compared with the untreated group.

Dipyridamole, an anti-platelet drug as a used for a secondary preventing and treating of stroke, has also an antiplasmodial activity and enhancer activity of chloroquine. The growths of *B. bovis* and *T. equi* were significantly inhibited at a 25 μM of dipyridamole, while *B. bigemina* and *B. caballi* were significantly inhibited at 1 and 10 μM of dipyridamole, on day 3 of cultivation, respectively. The IC_{50} of dipyridamole was calculated at 39.5±4.5, 26.3±10.9, 13.2±3.6, and 23.5±0.5 μM on growths *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively. In the viability assay, *B. bovis*, *B. bigemina*, and *T. equi* were failed to grow at the previous treatment of 100 μM of dipyridamole, while *B. caballi* was not grown at the previous treatment of 50 μM of of dipyridamole. As for in vivo inhibitory assay, dipyridamole could not inhibit *in vivo* growth of *B. microti* with at dose 100 mg/kg body weight treatment.

Clofazimine, currently used for treating leprosy for the human treatment. The present study shown that clofazimine was evaluated against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi in vitro* culture, and against *B. microti* in mice. The IC_{50} values of clofazimine against the *in vitro* growth of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 8.24±0.95, 5.73±0.57, 7.95±0.08, and 2.88±0.18 μM, respectively. In mice infected with *B. microti*, treatment with oral administration of 20 mg/kg clofazimine resulted in a significant lower peak parasitemia (1.6%) as compared to a control group (45.1%), which was comparable to subcutaneous administration of 25 mg/kg diminazene aceturate, the most widely used treatment for animal piroplasmosis. Although slight anemia was observed in both clofazimine- and diminazene aceturate-treated infected mice, the level and duration of anemia were lower and shorter than in untreated infected mice. Using blood transfusions and PCR, we also examined whether clofazimine completely killed *B. microti*. On day 40 post-infection, when the blood analysis was performed, parasites were not found in the blood smears; however, the DNA of *B. microti* was detected by PCR in the blood of clofazimine-treated animals and in several tissues of clofazimine- and diminazene aceturate-treated mice. The growth of parasites was observed in mice after blood transfusions from clofazimine-treated mice.

As for epidemiological study, I investigated *B. microti* infection in questing ticks in Mongolia. A total of 219 questing ticks were collected from three different Mongolian provinces (Bayan-Olgii, Khovsgol, and Selenge). Of these, 63 ticks from Selenge were identified as *Ixodes persulcatus*, while the remaining 156 (from all three provinces) were identified as *Dermacentor nuttalli*. When the tick DNA samples were screened using a *B. microti*-specific nested PCR, 19 (30.2%) of the 63 *I. persulcatus* ticks were found to be *B. microti*-positive. The parasite was not detected in *D. nuttalli*. Subsequently, the *18S rRNA*, *cox1*, and *tufA* sequences of *B. microti* were amplified, sequenced, and subjected to phylogenetic analyses. Sequencing analyses showed that the Mongolian *18S rRNA*, *cox1*, and *tufA* sequences were 99.6–100%, 96.7–97.2%, and 94.7–95.3% homologous, respectively, with *B. microti* R1 strain US-type sequences from humans. In the phylogenetic analyses, the Mongolian *cox1* and *tufA* sequences were found to be separate lineages, which formed sister-clades to the R1 strain sequences, while all of the Mongolian *B. microti 18S rRNA* sequences were clustered within US-type clade containing several other sequences of human origin.

B. venatorum, formerly known as *Babesia* sp. EU1, is a zoonotic hemoprotozoan parasites that commonly infects deer. I also investigated *B. venatorum* infection only in *I.*

persulcatus, an important tick vector capable of transmitting several tick-borne pathogens that cause babesiosis, encephalitis, tularemia, and Lyme diseases. DNA samples from questing *I. persulcatus* ticks (n=63) were again investigated that had been collected in Selenge province of Mongolia in 2012 and 2013 were screened for *B. venatorum* using a nested PCR assay. The findings showed that two of 63 DNA samples were positive for *B. venatorum*. The *18S rRNA* sequences amplified from *B. venatorum*-positive DNA samples shared high identity scores (96.1–99.9%) with known *B. venatorum* sequences derived from human and tick isolates. In phylogenetic analysis, the Mongolian *18S rRNA* sequences clustered with the previously characterized *B. venatorum* sequences.

In conclusion, methylene blue might be used for against bovine *Babesia* and equine piroplasma parasites while dipyridamole might not be used as a chemotherapeutic drug against these parasites, whereas clofazimine showed excellent inhibitory effects against *Babesia* and *Theileria* in vitro and in vivo, and further study on clofazimine is required for the future development of a novel chemotherapy with high efficacy and safety against animal piroplasmosis and, possibly, human babesiosis. In addition to reporting the presence of *B. microti* in ticks and *B. venatorum* in Mongolia for the first time, the present study identified *I. persulcatus* as a potential vector of this zoonotic *Babesia* in Mongolia, the present study found that Mongolian *I. persulcatus* ticks were infected with *B. microti* US-type and *B. venatorum*. These findings warrant large-scale studies to detect and characterize *B. microti* and *B. venatorum* in ticks, small mammals, deer and humans. Such studies should provide us with a better understanding of zoonotic *Babesia* epidemiology in Mongolia.