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Abstract of Dissertation

Applicant

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Title :

Studies on epidemiology, development of chemotherapy and genetic modification of piroplasmosis

(ピロプラズマ病の疫学, 薬剤開発および遺伝子改変に関する研究)

Abstract

Piroplasmosis is a disease caused by infection with apicomplexan intraerythrocytic parasites of the genera *Babesia* and *Theileria*. These are the most common parasites found in the bloodstream of mammals. Therefore, piroplasmosis is considered to be of significant economic, veterinary, and medical importance. This study investigated the prevalence of equine piroplasmosis in West Java, Indonesia, using serological and molecular diagnostic methods. To support the challenges of inadequate chemotherapeutic agents against piroplasm parasites, I evaluated novel therapeutic agents with high efficacy against piroplasm parasites by screening 400 compounds obtained from the MMV Pathogen Box against *Babesia* and *Theileria* parasites *in vitro* and *in vivo*. Furthermore, establishment of a gene modified *T. equi* was studied to study biology of parasites.

In chapter 1, I conducted surveillance of *T. equi* and *B. caballi* in horses reared in parts of West Java, Indonesia. Blood samples were collected randomly from 235 horses in four different districts (Bandung, Depok, Tangerang, and Bogor) in West Java. Of 235 surveyed animals, 5 (2.1%) and 15 (6.4%) were seropositive for *T. equi* and *B. caballi*, respectively, whereas 1 and 4 horses were nPCR-positive for *T. equi* and *B. caballi*, respectively. The *T. equi EMA-2* and *B. caballi BC48* gene fragments amplified by the nPCR assays were confirmed via bioinformatic and phylogenetic analyses. The *T. equi EMA-2* gene sequence from an Indonesian horse was identical to sequences from Florida and Washington strains and clustered together with these sequences in phylogeny. On the other hand, four Indonesian *BC48* gene sequences shared 99.8–100% identity scores. The present study is the first to report *T. equi* and *B. caballi* in horses in Indonesia. My findings highlight the need to monitor horses in Indonesia for clinical piroplasmosis caused by *T. equi* and *B. caballi*.

In chapter 2, I evaluated novel therapeutic agents with high efficacy against piroplasm parasites. In this study, the 400 compounds in the Pathogen Box provided by the Medicines for Malaria Venture foundation were screened against *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*. The initial *in vitro* screening performed using a 1 μM concentration as baseline revealed nine effective compounds against four tested parasites. Two hit compounds, namely MMV021057 and MMV675968, that showed an IC_{50} <0.3 μM and a selectivity index >100 were selected. The IC_{50}s of MMV021057 and MMV675968 against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 23, 39, 229, and 146 nM and 2.9, 3, 25.7, and 2.9 nM, respectively. In addition, a combination of MMV021057 and DA showed additive or synergistic effects against four tested parasites, while combinations of MMV021057 with MMV675968 and of MMV675968 with DA showed antagonistic effects. In mice, treatment with 50 mg/kg MMV021057 and 25 mg/kg MMV675968 inhibited the growth of *Babesia microti* by 54 and 64%, respectively, as compared to the untreated group on day 8. Interestingly, a combination treatment with 6.25 mg/kg DA and 25 mg/kg MMV021057 inhibited *B. microti* by 91.6%, which was a stronger inhibition than that by single treatments with 50 mg/kg MMV021057 and 25 mg/kg DA, which showed 54 and 83% inhibition, respectively. My findings indicated that MMV021057, MMV675968, and combination treatment with MMV021057 and DA are prospects for the further development of antipiroplasm drugs.

In chapter 3, I established a stable expression of GFP in *T. equi*. I selected an effective promoter of the *elongation factor-1 alpha* (*ef-1α*) gene. In this regard, I sequenced and identified the open reading frame of the *ef-1α* gene in *T. equi* (USDA strain) using RACE analysis. The mRNA sequence of the *ef-1α* gene in *T. equi* shared 98.3% identity with the sequence of *T. equi* (WA strain) retrieved from the GenBank and revealed the presence of two *ef-1α* orfs using a BLAST. Based on sequencing analysis, an intergenic region (IG) between the *ef-1α* orfs and the terminator from gDNA were amplified and sequenced. I designed a plasmid containing the IG of the *ef-1α* gene, a GFP–blasticidin (BSD) fusion gene, and a terminator fragment of the *ef-1α* gene. The designed plasmid was linearized and then transfected into the *ef-1α* gene locus of the *T. equi* genome using an Amaxa Nucleofector 2b device. Twenty-four hours after transfection, the cultures were treated with 10 × IC_{50} blasticidin for transgenic parasite selection. Expression of the green fluorescence parasite was observed within 24 h post transfection under a fluorescence microscope, and the GFP was consistently expressed for at least 2 months without drug pressure. PCR analysis revealed that the transfected plasmid integrated with the *ef-1α* genome, particularly in the B-direction gene of *ef-1α*. Moreover, the transfected *T. equi* grew normally and comparably with the wild-type parasite. This result is the first time a stable GFP-expressing *T. equi* has been established as a useful tool for understanding the biology of parasites and parasite–host interactions.