NRCPD-OUAVM Joint Research Report

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Project no: 2022-joint-19

1. Principal investigator

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Position: Research Scientist – Laboratory of Molecular Biology of Ticks (previously Laboratory of Vector Immunology)
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2. Project title:

Establishment of DiCre parasite lineages to study essential aspartyl peptidases of *Babesia*

3. Collaborating research group members at NRCPD

Name: Masahito Asada
Position: Associate Professor

4. Research period (in mm/dd/yyyy, and total number of years)

01/04/2022 -31/03/2023, one year

5. Purposes and objectives

The primary goal of this project is to develop innovative functional genomic tools for tick-borne Babesia parasites. Specifically, we aim to create a stable transgenic strain(s) of *Babesia* that expresses the DiCre recombinase. The DiCre conditional recombinase system allows for the functional analysis of essential parasite genes, which cannot be effectively studied using conventional non-inducible knock-out systems. Although this technique has been successfully employed in model species like *Toxoplasma gondii* and *Plasmodium falciparum* from the Apicomplexa group, it has not yet been introduced in *Babesia*.

The individual objectives of this project are as follows:

(i) Designing and cloning *Babesia* plasmid constructs that enable the integration of both Cre subunits into the same genomic locus of selected *Babesia* species.

(ii) Generating "parental" DiCre parasite line(s) and optimizing the transfection strategy for *Babesia*.

(iii) Implementing the loxP sites into the parasite using both episomal and intra-genomic approaches to confirm recombinase activity.

(iv) Conducting conditional knock-out experiments on selected *Babesia* target genes.

By achieving these objectives, we aim to advance our understanding of *Babesia* parasites and their associated diseases, ultimately contributing to the development of improved diagnostic and therapeutic strategies.

6. Outline of research process

During our work on this project and the visit of Dr. Sojka to NRCPD-OUAVM between November 6 and November 16, 2022, we prepared the initial version of the plasmid DNA vector containing the complete DiCre cassette for integration into the genome of our *Babesia divergens* strain. This construct was designed in such a way that by making only two changes in the homologous regions, we could use the same plasmid/DiCre cassette for integration into the genome of *Babesia bovis*, a related *Babesia* sensu stricto species that is of relevance to our Japanese collaborators in this project.

However, our subsequent attempts to electroporate and create *B. divergens* parental lineages through serial dilution revealed significant shortcomings in the initial design of the DiCre cassette-holding plasmid, leading to unsuccessful outcomes. Therefore, in 2022, we undertook a redesign and synthesis of a novel version of the DiCre cassette-holding plasmid to enable its integration into both *Babesia* species.

The work was also supported by our parallel CAS/JSPS joint mobility project, when two members of our team, Eliana Fernanda Galindo Cubillos (postdoc) and Ana Maria Osório De Barros De Almeida Filipe (PhD student), visited NRCPD in Obihiro for almost two months. During their internships they primarily focused on generating stable lines of *B. bovis* parasites that express the dimerizable Cre-recombinase (DiCre). This enzyme facilitates the conditional deletion of target genes, allowing for conditional knockdown (iKO). Both visiting researchers acquired practical skills related to the preparation of transgenic *Babesia* and subsequent analysis of the resulting phenotype. Additionally, they received intensive training in various knock-in/out techniques targeting specific genes in Babesia, which are crucial for studying gene function in this organism.

The main outcome of their two-month internship was the generation of transgenic strains of *B. divergens/ B. bovis* carrying the updated version of the DiCre cassette. These strains are currently undergoing cloning by serial dilution and analysis through PCR.

Furthermore, our collaboration with the University of Geneva, Switzerland, regarding recombinant expression, purification, and biochemical characterization of two BdASP3 proenzymes prepared in baculovirus-infected Sf9 insect cells, has continued. Results from this collaboration were presented by team members Sojka, Jalovecká, and Šnebergerová at the International Congresses of Parasitology – ICOPA XV, held in Copenhagen, Denmark, from 21-26 August 2022.

7. Outline of research achievements

- *B. divergens* and *B. bovis* were selected as model organisms.
- Specific promoters for *B. divergens/B. bovis* were identified and their sequences were determined.
- Specific 3' untranslated regions (UTRs) for *B. divergens/B.bovis* were identified and their sequences were determined.
- The sensitivity of *B. divergens* to Blasticidin-S-Deaminase (BSD) and WR99210 selection markers was validated.
- The first and second generation of a plasmid DNA vector containing the full DiCre cassette for incorporation into the genome of *B. divergens/B. bovis* was designed and synthesized.
- Team members were intensively trained in strategies to perform knock-in/out techniques on specific genes in *Babesia* by NRCPD-OUAVM host lab members.
- Transgenic strains of *B. divergens/B. bovis* carrying the updated version of the DiCre cassette were generated. This process involved cloning by serial dilution and integration analyses through PCR.
- Aspartyl proteases recBdASP3a/b, encoded by first-choice DiCre recombinase target genes, were expressed in both *E. coli* and Sf9 insect cells. These expressed proenzymes were then biochemically characterized using Western blots and immunomicroscopy techniques. Antibodies raised against recBdASP3a/b were utilized for these analyses.

8. Publication of research achievements

Due to the COVID-19 pandemic and the closure of Japan's borders for tourism and business travels, the internships of CZ team members in Japan were initially made available only in late 2022. Consequently, there hasn't been sufficient time to publish the obtained results before the deadline of this report. However, we plan to publish the results of the ongoing collaboration between the CAS and NRCPD-OUAVM teams in 2023-2024. These publications will be based on our long-term collaboration, supported by other concurrently running projects.

Attach reference materials as necessary.