NRCPD-OUAVM Joint Research Report

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Project no: 2024-joint-7

1. Principal investigator

Name: RNDr. Daniel Sojka, Ph.D.

Position: Research Scientist – Laboratory of Molecular Biology of Ticks

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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: Dr. Masahito Asada

Position: Associate Professor

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

01/04/2024 -31/03/2025, one year

5. Purposes and objectives

The 2024 NRCPD-OUAVM Joint Research Proposal sought to expand on our prior collaborative efforts (2019, 2022, 2023, 2024), where we successfully developed transgenic *Babesia bovis* and *Babesia divergens* parasites expressing DiCre recombinase. This technology facilitates the functional analysis of essential parasite genes, previously demonstrated in model species such as *Toxoplasma gondii* and *Plasmodium falciparum*. The 2024 proposal aimed to leverage the stable DiCre parasite lineages of *B. bovis* and to perform conditional -inducable- knockouts (cKO or iKO) of aspartyl proteases BdASP3a (023140) and BdASP3b (006490) of the C clade of apicomplexan aspartyl proteases, which are analogous to plasmepsins X/IX for *P. falciparum* and thus represent the potential master regulators involved in invasion and egress during *Babesia*'s asexual blood stages. Project aimed at phenotypical characterization of the ASP3a/b deleterious phenotypes, with the aim to validate the critical roles of these enzymes in the erythrocytic lifecycle of *Babesia*. This work builds up on our preliminary findings with plasmepsin X/IX and TgASP3 specific inhibitor 49c, supporting Bd/BbASP3 as promising drug targets for *Babesiosis* control.

6. Outline of research process

In accordance with the proposed plan, we utilized the extended project timeline to generate cloned populations of DiCre-expressing *B. bovis* and *B. divergens*. During the course of this work - including Dr. Dede's research visit to NRCPD-OUAVM from February 21 to March 18, 2025 - we successfully confirmed DiCre integration in *B. bovis*. Following the visiting, we also verified the presence of DiCre in *B. divergens*.

Both species were targeted using the same gene of interest (GOI) for homologous recombination; however, in *B. divergens*, the selected GOI unexpectedly impaired parasite growth and disrupted the blood cycle stages. As a result, despite obtaining a DiCre-integrated clone, we must now select an alternative GOI and generate new DiCre lines.

In parallel, we prepared control GFP/mCherry plasmid vectors for episomal monitoring of LoxP site excision by DiCre recombinase in *B. bovis*. Transfection of these plasmids into *B. bovis* is currently underway to validate Cre/LoxP excision efficiency.

The 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 *B. divergens*, a related *Babesia* sensu stricto species that is of relevance for us back in the Czech laboratory at IoP BC CAS. PCR confirmed the correct integration of plasmids into parasite genomes, and expression of DiCre recombinase subunits was confirmed via quantitative PCR. Additionally, control GFP/ mCherry holding plasmid vectors were prepared for episomal control of LoxP site excision by the DiCre recombinase in *B. bovis* and the Cre/Lox excisions are currently being confirmed by transfection of these plasmids to *B. bovis* DiCre lineages. We are currently in the process of designing a *B. divergens* expression plasmid based on the established *B. bovis* vector system, with necessary adaptations to ensure compatibility with *B. divergens* biology and expression requirements.

The study also targeted *B. bovis*, a key pathogen inducing bovine babesiosis in NRCPD-OUAVM. Dr. Asada's group transfected *B. bovis* with two plasmids: one has the DiCre recombinase and the other has LoxP (GFP-mCherry) sites respectively. In Dr. Alper Dede's stay, successful integration of these genetic elements was partially monitored and validated using the Indirect Fluorescent Antibody Test (IFAT) and specific Polymerase Chain Reaction (PCR) assays. The precise placement of LoxP sites were confirmed. Additionally, the regions with targeted primers were proved and, will be prepared for rapamycin-induced excision verification (still ongoing to detect the correct drug concentration). Dr. Dede collaborated on the design and preparation of LoxP holding plasmid cassettes and the preparation of transgenic *B. bovis.* It was also decided that plasmids would be constructed following the design: 5'HR-LoxP-Gene of Interest (GOI)-Myc-Terminator-BgActin-BSD-LoxP-3'HR. He contributed to phenotypic analysis and transgenic *Babesia* isolation. He participated in discussions on the knock-in and knock-out technologies that target specific genes, for instance, those in heme and proteolytic pathways, to identify their essential roles in *Babesia* spp. These efforts advanced understanding a clearer insight into transgenic parasite preparation and supported the objectives for the experimental goals of the Sojka lab.

Furthermore, the live imaging of the parasites that contain mCherry gene enabled us to observe gene expression in real time and successful transfection. A substantial improvement towards conditional gene manipulation in *B. bovis*, these cultures were maintained under drug selection or induction conditions to maintain the DiCre-LoxP system activity.

In addition, we successfully continued the characterization of the two BdASP3 isoenzymes, which were recombinantly expressed in *E. coli* DE3 as C-terminal 6x(His)-tagged proteins in an insoluble form. This approach, which replaced the initially planned expression in baculovirus-infected insect cells due to issues with low yields and protein instability, has proven effective. The *E. coli*-expressed BdASP3 proteins were purified under denaturing conditions and subsequently refolded via step-down dialysis from 8 M urea buffers. The refolded proteins were confirmed to be catalytically active and are currently used in kinetic assays with fluorescent peptidyl substrates. In addition, we have successfully generated inactive mutant variants of both BdASP3 isoenzymes by substituting a key aspartic acid residue with alanine. These mutants are currently being used in large-scale production aimed at obtaining high-quality crystals for structure determination via X-ray crystallography. All the results obtained during 2023–2024 will be included in a forthcoming publication planned for submission to a high-impact journal by the end of 2025, with Pavla Šnebergerová as the first author and Dr. Sojka as the last and corresponding author.

Our findings were presented at several international conferences in 2024 and 2025. Dr. Sojka made two active contributions at the 15th International Symposium on Ticks and Tick-borne pathogens TTP 11th in Cuba, presenting on 'Proteases associated with the apical complex of Babesia" and International Babesiosis Meeting 2025 presenting "Proteases driving the apical complex of Babesia during egress and invasion of host red blood cells.

7. Outline of research achievements

- Successfully obtained cloned populations of DiCre *B. bovis* and *B. divergens*, but *B. divergens* need a different design.
- We PCR confirmed their integration to the parasite genome
- Lox-flanked GFP/m mCherry holding plasmid vectors for episomal control of LoxP site excision by the DiCre recombinase in *B. bovis*
- We designed the final plasmid for BdASP3 cKO using the DiCre approach.
- Live imaging of *B. bovis* with mCherry was achieved.
- In parallel, we successfully expressed BdASP3a/b isoenzymes as C-terminal 6x(His)-tagged proteins in *E. coli* and we are using them for 3D structure studies using protein crystallography.

8. Publication of research achievements

Some of the experience gained during this project contributed to the study published by Robbertse et al. (2024), titled “Evaluating Antimalarial Proteasome Inhibitors for Efficacy in Babesia Blood Stage Cultures” in ACS Omega (PMCID: PMC11561622, PMID: 39554424).

9. Future goals

Our future goals include obtaining *B. divergens* DiCre and LoxP clones carrying mCherry and BSD resistance markers, followed by confirmation of successful transfection using IFAT, PCR integration, and flow cytometry. The final plasmid for the conditional knockout (cKO) of BdASP3, containing a floxed BdASP3 gene sequence, has been designed and will be transfected upon completion of Cre/Lox episomal excision validation. The resulting cKO BdASP3a/b lines will enable rapamycin-induced gene excision and phenotypic analysis. This approach will also be used to monitor the effects of other genes of interest (GOIs) on parasite development. Importantly, this cKO system represents a valuable tool for the *Babesia* research community, enabling functional studies of essential genes—many of which encode high-priority enzymatic targets for future therapeutic development.