NRCPD-OUAVM Joint Research Report

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1. Principal investigator

Name: Albert Mulenga

Position: Professor of Veterinary Parasitology

Affiliation: Texas A&M University

2. Project title:

Establishment of split Cas9 for functional characterization of essential genes in *Babesia bovis*

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

We aimed to establish an inducible CRISPR/Cas9 system for *B. bovis* to be used for functional characterization of essential genes in this parasite.

6. Outline of research process

The workflow for adaptation of inducible CRISPR/Cas9 consists of the following aims:

1. Construction of splitCas9-expressing plasmid
2. Generation of splitCas9-expressing *B. bovis*
3. Optimization of rapamycin to be used for reassembly of Cas9 fragments
4. Validation of CRISPR/splitCas9 for induction of gene knockout

7. Outline of research achievements

A plasmid construct was generated to express splitCas9 and inserted into *ef-1α* locus of *B. bovis* genome. PCR assay confirmed the insertion of the plasmid construct into the target locus. To recycle the selection marker, yFCU gene was used in the expression plasmid which confers sensitivity to 5-fluorocytosine (5-FC). A growth inhibition assay was conducted using the transgenic parasite to find the optimum concentration of 5-FC for negative selection. The calculated EC_{50} was 26±1.2 nM for transgenic parasite expressing splitCas9 while no inhibition was seen in wildtype parasite. We used 1μM of 5-FC for negative selection which resulted in selection of parasites that lost the selection marker.

Next, we measured IC_{50} of rapamycin to optimize the concentration that can be used for Cas9 assembly. A growth inhibition assay was done using wildtype parasite. The calculated IC_{50} was 13.9±11.6 µM and no inhibition was seen at 1µM which was decided to be used for Cas9 assembly.

To validate CRISPR/splitCas9 for induction of a gene knockout, we targeted BbVEAP (VESA1-export associate protein) which previously was shown to be essential for the parasite growth *in vitro* [1]. A circular plasmid expressing guide RNA and having homologous arms for *bbveap* locus repair was transfected into splitCas9-expressing parasite. Following appearance of transgenic parasites, using PCR we found that *veap* locus was modified before the addition of rapamycin. This could be due to the assembly of Cas9 peptides which may happen during protein synthesis and transport in the cytoplasm. In our construct design, we fused Cas9 C-terminal peptide with a nuclear localization signal which will transport the peptide to the nucleus. Cas9 N-terminal peptide has no signal and is assumed to remain in the cytoplasm. Since Cas9 peptides are expressed using strong *ef-1α* promoter, abundant Cas9 peptides may be assembled and exported to nucleus where the functional holoenzyme produces double-strand break in *bbveap* locus. Recently it was shown in *Toxoplasma gondii* that the addition of nuclear export signal to Cas9 N-terminal may prevent this unwanted assembly before addition of rapamycin [2]. We are planning to modify splitCas9-expressing plasmid by fusing nuclear export signal to Cas9 N-terminal peptide and perform the experiments again.

Altogether, we were able to achieve 3 aims mentioned in the research outline: construction of splitCas9-expressing plasmid, generation of splitCas9-expressing *B. Bovis*, and optimization of rapamycin to be used for reassembly of Cas9 fragments. The final aim could be achieved by modification of splitCa9-expressing plasmid in future studies.

8. Publication of research achievements

None.

Attach reference materials as necessary.

1. Hakimi, H., et al., *Novel Babesia bovis exported proteins that modify properties of infected red blood cells.* PLoS Pathog, 2020. **16**(10): p. e1008917.
2. Li, W., et al., *A splitCas9 phenotypic screen in Toxoplasma gondii identifies proteins involved in host cell egress and invasion.* Nat Microbiol, 2022. **7**(6): p. 882-895.