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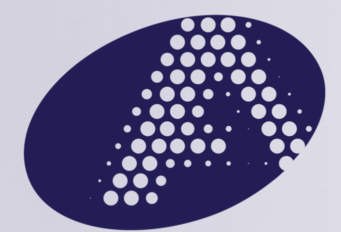

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CRISPR/Cas9-Engineered Fluorescent Reporter Lines of *Babesia duncani*

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Abstract

CRISPR-Cas9 technology is a valuable tool for gene editing in various apicomplexan parasites, including *Plasmodium falciparum*, the causative agent of human malaria. However, there is a dearth of efficient CRISPR-based genetic modification tools for *Babesia duncani*, a related apicomplexan parasite responsible for human babesiosis, an emerging tick-borne disease. Such tools could play a pivotal role in probing gene function and identifying new targets for drug and vaccine development. The availability of an established in vitro culture system for *B. duncani* and a published genome offers a promising opportunity to develop an efficient gene editing system in this parasite. We leveraged advances in genome modification protocols for bovine *Babesia* parasites and *P. falciparum* to create *B. duncani* lines that stably express GFP or mCherry for a variety of downstream applications, including compound screening. To achieve this, we developed a Cas9/gRNA expression plasmid that is codon-optimized for *Babesia* parasites. This plasmid expresses Cas9 from *Streptococcus pyogenes* and a guide RNA scaffold into which target-specific gRNAs can be cloned. To examine the practical utility of this Cas9/gRNA expression vector, gRNAs specific for the parasite apical membrane antigen 1 (AMA-1) or thioredoxin peroxidase 1 (TPX-1) were cloned into this vector. The resulting plasmid and a donor DNA plasmid expressing either mCherry-tagged AMA-1 or GFP-tagged TPX-1 were co-transfected into *B. duncani* parasites by electroporation. *B. duncani* parasites expressing GFP or mCherry were observed 14 days after the transfection, with 100% editing efficiency. These results highlight the practical utility of this gene editing system. This work will contribute towards the identification of novel targets for drug and vaccine development, and a better understanding of the biology of *Babesia* parasites that affect humans.

Background

- Babesiosis is a malaria-like disease caused by Babesia parasites that are transmitted to humans through bites from infected ticks (Figure 1).
- There are over 100 known Babesia species, but the major causative agents of human babesiosis are *B. microti, B. divergens,* and *B. duncani.*
- A limited number of drugs is available for the treatment of human babesiosis, but there is no suitable vaccine for the disease.
- Understanding gene function is critical for developing new drugs and vaccines for human babesiosis, but there are no efficient tools to do so.

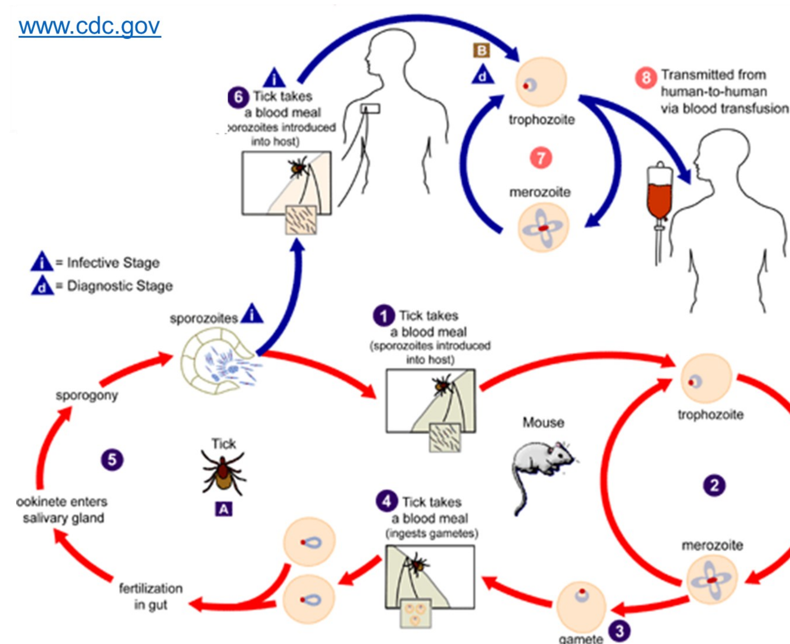

Figure 1: Life cycle of human babesia parasites. Humans are incidental hosts; primary hosts include a rodent and a tick of the *Ixodes* genus. A human being is infected through the bite of an infected tick (for example, while hiking). During a blood meal, the tick inoculates *Babesia* parasites (sporozoites) into erythrocytes (*6*) where they undergo differentiation and continuous rounds of asexual replication (*7*), leading to clinical symptoms such as fever, chills, fatigue, and hemolytic anemia. Humans are dead-end hosts. Adapted from CDC: https://www.cdc.gov/dpdx/babesiosis/index.html

Technical Approach

Project goal: To develop CRISPR/Cas9 genome modification tools for human *Babesia* parasites that can be used to probe gene function.

Specific objective: To adapt genetic manipulation protocols routinely used for *P. falciparum* and bovine *Babesia* parasites to create *Babesia duncani* reporter lines expressing green fluorescent protein (GFP) or the red fluorescent protein, mCherry, for use in screening candidate drug compounds for activity.

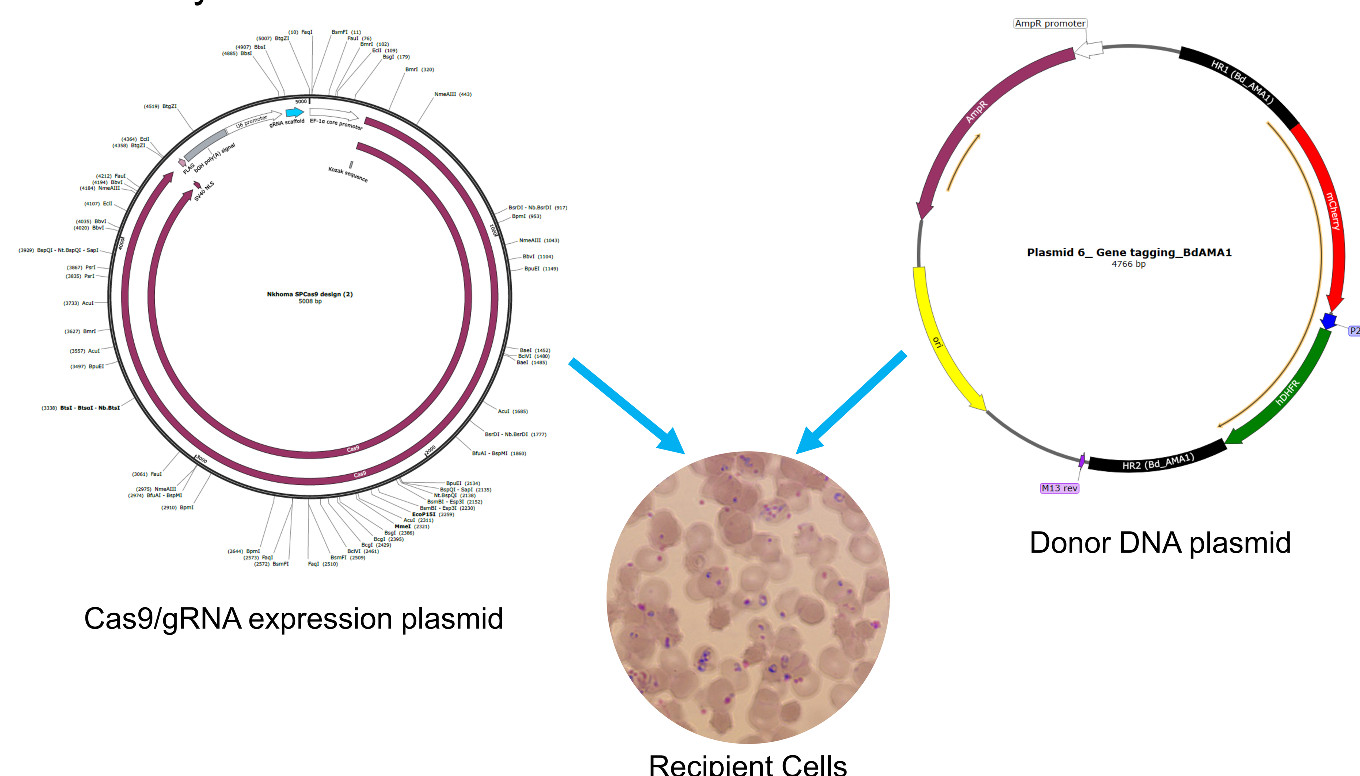

Figure 2: Genome modification in *Babesia duncani*. To introduce a GFP-tagged or mCherry-tagged gene into *B. duncani* parasites, gRNAs specific for either the thioredoxin peroxidase 1 (TPX-1) or apical membrane antigen 1 (AMA-1), respectively, were cloned between the two BbsI sites in the Cas9/gRNA expression plasmid (plasmid image on the left). The resulting plasmid was expanded using chemically competent cells and validated using next-generation sequencing. Donor plasmids containing GFP-tagged TPX-1 and mCherry-tagged AMA-1 (plasmid image on the right) were obtained from our collaborators. Both plasmids were introduced into a cloned *B. duncani* parasite (BEI Resources catalog number NR-59103) by electroporation, followed by 10 days of selection with the antifolate drug WR-99210. Transfected parasites were recovered three days after the cessation of drug selection and cultivated in drug-free media.

Parasites transfected with a donor plasmid containing a GFP-tagged TPX-1 gene

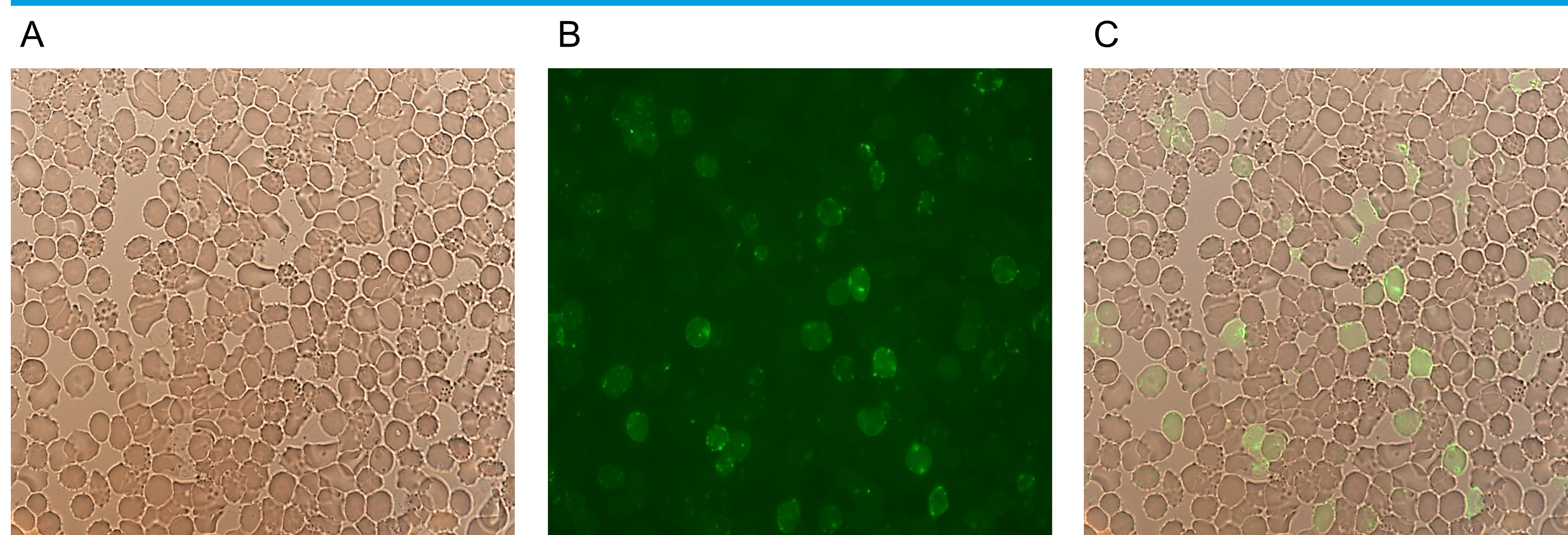

Figure 3: Successful transfection of a GFP-tagged gene into the *B. duncani* genome. Thin blood smears were prepared from transfected cells 14 days after the cessation of drug selection. Slides were visualized using a fluorescence microscope (wet mount, 100x magnification). (A) A bright field image was merged with the (B) GFP image to better visualize the parasitized cells. (C) In the merged image, parasitized cells expressing GFP were visible. The GFP signal in these parasites remained stable after several cycles in culture and following cryopreservation of the cells and subsequent re-growth from frozen vials.

Parasites transfected with donor plasmid containing mCherry-tagged AMA-1 gene

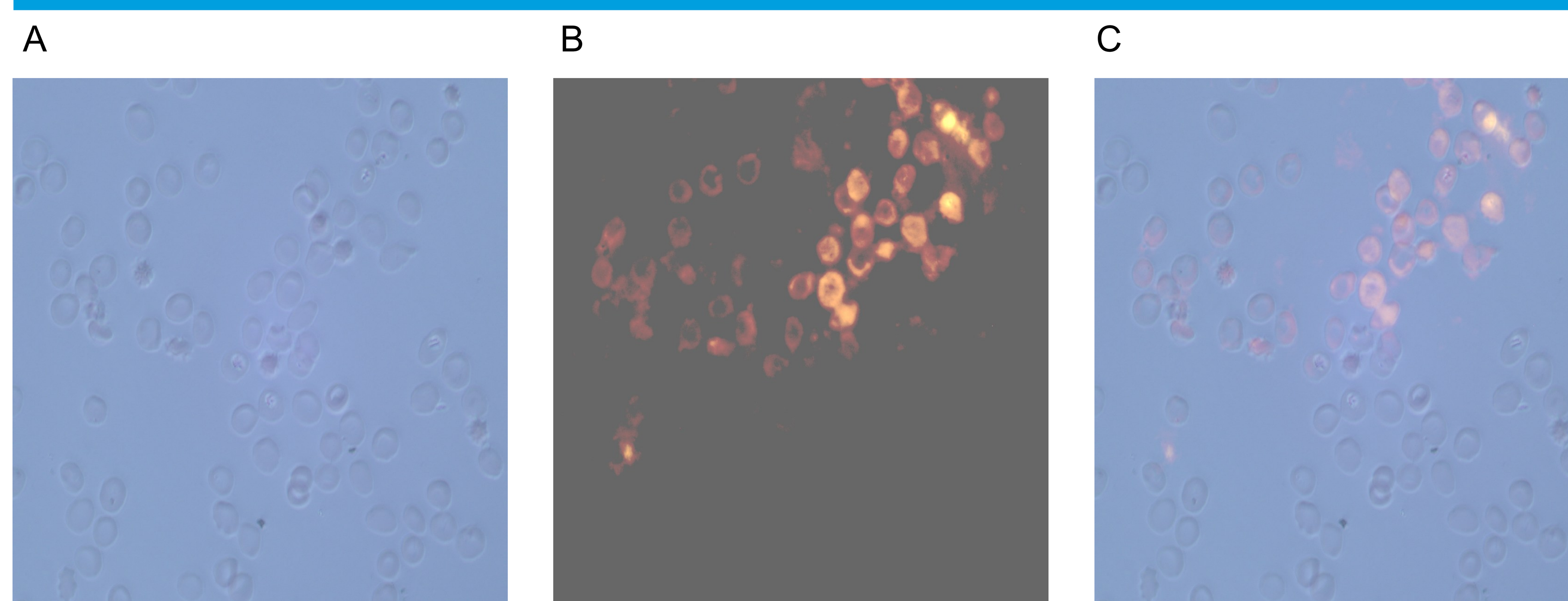

Figure 4: Visualization of parasites transfected with an mCherry donor plasmid. (A) A bright field image was merged with the (B) red fluorescent image to better visualize the parasites. (C) The merged image shows parasitized erythrocytes with red fluorescence signal, indicating successful transfection of mCherry-tagged AMA-1 into the progenitor parasites.

Summary

- We have developed a codon-optimized Cas9/gRNA expression plasmid that can be utilized for editing different *B. duncani* genes to probe their functions.
- We have successfully adapted gene modification protocols previously used in *B. bovis* and *P. falciparum* experiments to perform gene editing in *B. duncani.*
- We have utilized these protocols to create *B. duncani* GFP- and mCherry-expressing lines, which can be used for various applications, including compound screening.

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Planned Future Work

- Perform immunofluorescence assays using anti-GFP and anti-mCherry antibodies to amplify the fluorescence signal and enhance visualization of parasitized cells.
- Conduct whole-genome sequencing of both the progenitor *Babesia* parasite (BEI Resources catalog number NR-59103) and the transfected parasite lines to confirm integration and examine off-target effects.
- Evaluate the potential use of GFP and mCherry-expressing lines for in vitro and in vivo compound screening.

Acknowledgements

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