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Development of Needed Tools for Babesia duncani, a Rising Cause of Human Babesiosis

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DEVELOPMENT OF NEEDED TOOLS FOR *BABESIA DUNCANI*, A RISING CAUSE OF HUMAN BABESIOSIS

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Abstract

Human babesiosis is a bloodborne, tick-transmitted zoonotic disease that has seen an increase in cases across the United States and Canada. *Babesia duncani* is the second most prevalent and most virulent cause of human babesiosis but little is known of its cellular biology, molecular pathogenesis, and molecular genetics. To remedy this and help accelerate research on *Babesia duncani*, we worked towards developing a functional Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system for *Babesia duncani* while identifying strong promoters to drive expression of genes, developing an optimized transfection protocol, and identifying suitable selectable markers.

To identify strong promoters to drive expression of genes in transfections, reverse transcriptase (RT)-qPCR was performed on RNA extracted and synthesized into cDNA. To find a workable transfection protocol that could be optimized, established *Babesia* and *Plasmodium* transfection protocols from the literature were screened for their transfection efficacy (amount of *nanoluciferase* transcript produced) and impact on parasite viability following transfection by RT-qPCR readout. Once a workable transfection protocol had been identified, the protocol was optimized by varying the number of parasites and amount of DNA used per transfection. A CRISPR/Cas9 transfection was performed using the identified optimized transfection protocol. Lastly, to identify suitable selectable markers a novel 384-well high throughput compound screening format was developed. *Babesia duncani* were incubated for 48-hours with the compound of interest and lysed in the presence of propidium iodide before fluorescence was read 24-hours later.

Histone 2B (*H2B*) promoter was identified as the strongest promoter of those tested. *Enolase* and *elongation factor-1α* (*eF-1α*) were the next strongest promoters but had roughly 3- and 5-fold less expression than *H2B*, respectively. Govindarajalu et al, 2019 Lyse-Reseal protocol gave the third highest amount of *nanoluciferase* transcript without negatively impacting parasite growth following transfection. It was selected as the protocol to be optimized, with results indicating increased number of parasites and increased amount of DNA used per transfection led to greater transfection efficiency. However the results also indicated there may also be an inherent limitation to the maximum efficiency obtainable with the protocol seen in the diminishing return when increasing the number of parasites or amount of DNA used. The results of CRISPR/Cas9 transfection were promising as a mCherry expressing *Babesia duncani*, the expected outcome, was observed 24-hours post transfection however no other ones were observed following that. Using the high throughput compound screening format a handful of compounds were tested, identifying potential novel selectable markers for *Babesia duncani* as well as avenues for drug development.

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Foremost I would like to acknowledge and thank Chris Huston for allowing me to become a member of his laboratory all those years ago (5 years but it is not like anyone has been counting). I couldn't have gotten to where I am today without him and I certainly wouldn't be the scientist (in training) I am today without him, his laboratory, and the wonderful people he has chosen to be in his laboratory. Speaking of those wonderful people, I can't thank Bethany Korwin-Mihavics, Ethan Mattice, and Jubilee Ajiboye enough for taking me under their wings as Ph.D. students, teaching me anything and everything, always answering my questions (no matter how stupid the question seemed to me), giving me valuable feedback, being an amazing support system and voices of reason. For many of the same reasons, I also can't thank Jose (Ze) Teixeira and Peter Miller enough as their expertise was invaluable when I hit dead ends or difficulties in my research (which is often in science). I also must give Ze a special mention as (whether he wanted to or not), he taught me everything when I first joined the lab and was the real push behind putting the 384-well high throughput screening format into the assay format.

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Introduction

The apicomplexan parasites of the genus *Babesia* cause a bloodborne, malaria-like infection, known as babesiosis, in a wide variety of animals[1]. *Babesia* species were first identified by Victor Babes in 1888 as the causative agent of febrile hemoglobinuria in cattle[2]. A few years later in 1893, two researchers identified ticks, now known to be *Ixodes* species[1], as the vector for *Babesia* species[3]. Until the 1950s-1960s, babesiosis was thought to be a disease exclusive to animals with cases primarily being identified in livestock, such as cattle[1], sheep[1], and wildlife, such as rodents[4], and cervids[5]. In 1957 a novel piroplasmas agent was identified in a splenectomized Croatian herdsman[6]. Diagnostic testing ruled out malaria and other known human piroplasmas agents, leading the researchers to attribute the disease to *Babesia bovis,* which was present in some of the herdsman's cattle[6]. Twelve years later in 1969, several immunocompetent individuals on Nantucket Island, Massachusetts were afflicted with an infection caused by a likewise unknown piroplasmas agent that was designated *Babesia microti*[7].

Since 1969, the incidence and geographic distribution of human babesiosis in the continental United States has increased dramatically[8]. This observation can be attributed to increased awareness and diagnostic capabilities, increase in the tick's range and population size, and identification of new disease causing *Babesia* species in humans[8]. One such species was *Babesia duncani*, first identified in 1991 in an immunocompetent Washington state resident[9]. Since the identification of *Babesia duncani,* cases of human babesiosis caused by it have been reported all along the western coast of the United States and later in Canada, with reported cases increasing in both number and geographic range as the years have progressed[10,11]. This knowledge prompted healthcare officials to designate babesiosis as a nationally notifiable disease in 2011[12] stirring interest in research on *Babesia* species that infect humans.

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Literature Review

Apicomplexa refers to a large phylum of, mainly, parasitic protists with an apical complex of organelles involved in cell invasion[1]. The phylum is large, infecting a diverse range of vertebrate hosts, and can be divided into four subgroups (depending on who is asked)[2]. Those four subgroups are: coccodia, which includes *Cystoisospora, Eimeria*, and *Toxoplasma*; gregarines, which include *Cryptosporidium*; haemosporidia, which includes *Babesia* and *Plasmodia;* and marosporida[2]. Similar to Apicomplexa, *Babesia* refers to a large family of parasites that infect a diverse range of vertebrates, from birds, cats, cows, and dogs, to humans[3]. *Babesia* are unique in the Apicomplexa in that they infect the red blood cells (RBC) of their host in their asexual life stages and ticks in their sexual life stages[3]. Occasionally, humans can become infected after being bitten by a tick carrying *Babesia divergens*, *Babesia duncani*, or *Babesia microti*, in addition to their primary vertebrate hosts[3]. *Babesia duncani* and *Babesia microti* are the most prevalent causes of human babesiosis but are the most phylogenetically seperated[3] from other heavily researched *Babesia* spp., mainly *Babesia bovis* and *divergens*.

This poses a problem as *Babesia duncani* is the second most prevalent cause of human babesiosis in the United States[4,5,6,7], and the most virulent *Babesia* species[8,9], necessitating effective therapies but it is unclear if any of the research on *Babesia bovis* or *divergens* will translate to *Babesia duncani.* As such, little is known about the cellular biology, molecular pathogenesis, and molecular genetics of *Babesia duncani*.

Prior to 2018, studying *Babesia duncani* was hindered by a lack of a continuous *in vitro* culture system, with cultivation relying on the inoculation of mice and hamsters[10]. A continuous *in vitro* culture system was developed by Abraham and colleagues which they utilized to determine the effective concentrations (EC_{50}) of the current recommended therapies, either atovaquone and azithromycin or clindamycin and quinine[8]. The development of the *in vitro* culture system has

largely stimulated interest in the development and screening of novel and old small molecules for anti-babesial activity. Any interest in the cell biology, microbiology, and molecular genetics of *Babesia duncani* has been framed in the context of drug development, such as Virji and colleagues[11] sequencing and annotating the apicoplast and mitochondrial genomes of *Babesia duncani* searching for potential drug targets.

It wasn't until 2022 that two research groups sequenced and annotated the nuclear genome of *Babesia duncani*[12, 13]. Independent of these two groups, another group successfully demonstrated transient and stable transfection of *Babesia duncani* utilizing human dihydrofolate reductase (hDHFR) as the selectable marker and eGFP as the reporter[14]. Similar transfections of *Babesia gibsoni*[15], *sp. Xinjiang*[16], *ovata*[17], *microti*[18] and *bovis*[19] have been reported over the past 20 years. Despite the success of transfection in the *Babesia* species listed above, Clustered Regularly Interspaced Short Palindromic Pepeats (CRISPR)/Cas9 systems have only been demonstrated in *Babesia bovis*[20] and *divergens*[21], with no known reports of a CRISPR/Cas9 system in *Babesia duncani*. These CRISPR/Cas9 systems used a donor template that is integrated after Cas9 creates a double strand break in the DNA as *Babesia* spp. lack non-homologous end joining[20,21]. The lack of a CRISPR/Cas9 system in *Babesia duncani* is an impediment to the study of the cellular biology, molecular pathogenesis, and molecular genetics of *Babesia duncani*.

The main aim of this thesis is to fill in this gap by working toward a CRISPR/Cas9 system for *Babesia duncani,* with the sub aims of identifying strong promoters to drive expression of genes, developing an optimized transfection protocol, and identifying suitable selectable markers for use in future *Babesia duncani* research.

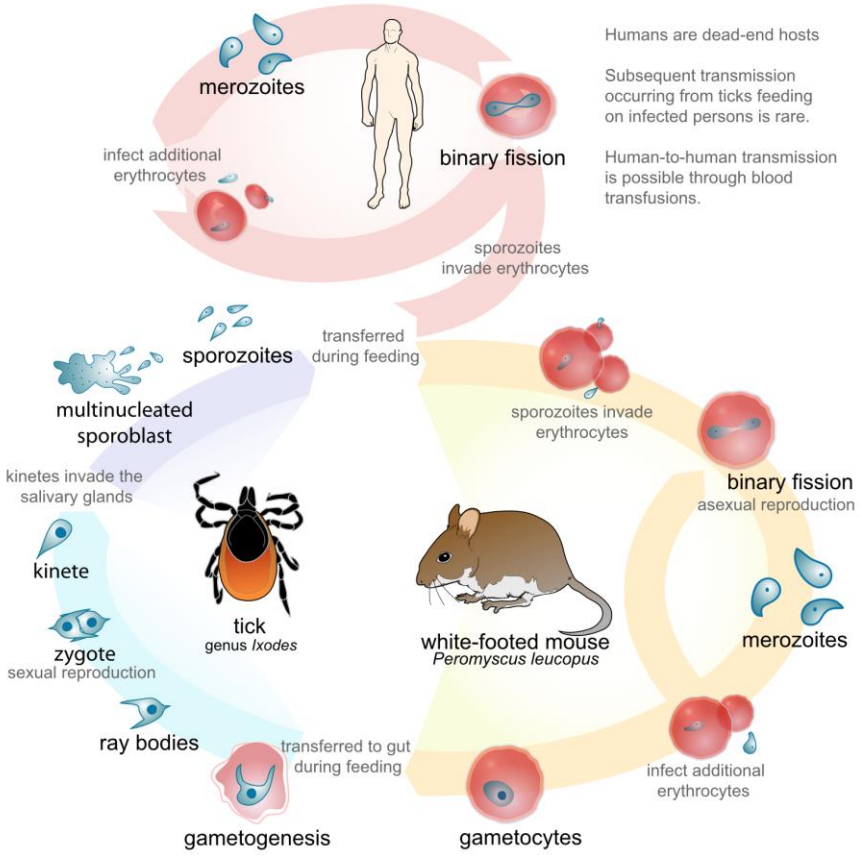

Figure 1. Generalized Lifecycle of *Babesia* spp. that Infects Humans. The figure specifically shows the lifecycle of *Babesia microti* as it infects the white-footed mouse in its asexual life stages and an unknown ixodes spp. tick in its asexual life stage. Note: By Villarreal, M. R. (2008). *Life cycle of the Parasite Babesia, (B. microti or B.d ivergens) including the infection to humans.* [Digital]. Wikipedia.

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Chapter One – Identification of Strong Promoters

Introduction

Successful endogenous gene expression of exogenously introduced DNA, such as a plasmid, can rely heavily on the promoter driving the gene of interest on the plasmid once it is successfully introduced into the organism. In the case of a Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 system, constitutive expression is required of both genes encoding the CRISPR guide RNA and Cas9 enzyme respectively to ensure ample opportunities for gene editing events prior to the loss of the transient plasmid encoding the CRISPR/Cas9 system or application of drug selection. This constitutive expression requires what are termed "strong promoters" capable of constantly driving transcription of its gene, no matter what point in the life cycle the cell is in. Typically, these are thought of as housekeeping genes and/or essential genes to the cell's function, although this is not always the case.

There are multiple ways of identifying these "strong promoters", with the simplest being reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). RT-qPCR has been used previously to identify "strong promoters" in a wide variety of organisms, such as bacteria[1], insects[2], and yeast[3]. Additionally, while ultimately less informative than other approaches on novel "strong promoters", such as RNA-seq, RT-qPCR allows directed testing of "strong promoters" from related species, such as *Babesia* spp. or other Apicomplexa. This is advantageous for newly or under-researched agents, such as *Babesia duncani*, as it focuses the scope of genes to be tested.

The current study was conducted to assess the strength of selected genes, who are known to have "strong promoters" in other *Babesia* or Apicomplexa spp., in *Babesia duncani*. The primary outcome measured was cycle threshold (Ct) that was normalized to the Ct of *elongation factor-1α* (eF1-α) to give -ΔCt.

Methods

Culture Maintenance

The culturing of *Babesia duncani* was essentially as described in Singh et al. 2022[4] with a few modifications; Media, completed DMEM/F12 (cDMEM; 20% heat-inactivated fetal bovine serum (Sigma-Aldrich catalogue #12306C-500ML), 1x HT media supplement (Sigma-Aldrich catalogue # H0137-10VL), 1x L-glutamine (Gibco, catalogue # 25030-081), 1x antibiotic-antimycotic (Gibco catalogue #15240-062), and 1x gentamicin (Gibco catalogue #15710-072)), was changed every other day and cultures were kept in a modular incubator gassed with 2% O_2, 5% CO_2, 93% N_2 at 37ºC. Passage occurred once a week into fresh blood at 1-2% parasitemia, 5% hematocrit and fresh blood was obtained as needed for experiments/passages from the University of Vermont Medical Center Blood Bank.

Parasitemia for passaging was determined using Giemsa staining and light microscopy. Briefly, thin blood smears were prepared and fixed by 100% methanol for 2 minutes then allowed to airdry. Once dry, the slides were stained in a solution of .02% Giemsa stain solution (Electron Microscopy Sciences, catalogue #6153) and 1% phosphate buffer solution (PBS), pH 7.2 for 10 minutes. After 10 minutes, each slide was dipped in deionized water eight times then allowed to airdry on a slant. A minimum of 500 red blood cells, in groups of 100, were counted and parasitemia was reported as number of infected red blood cells divided by total of red blood cells counted (both infected and uninfected).

Trizol-Chloroform RNA Extraction

RNA was extracted using a modified protocol of Sue Kyes et al., 2000[5]. Briefly, 1200µL of freshly washed blood was infected with 400µL of highly parasitized blood and brought to 5% hematocrit with cDMEM and then split into two T25 flasks. Media was changed every other day and on day 5 post infection, the first culture was harvested and washed three times with 1x

phosphate buffer solution (PBS), no Ca^{2+} or Mg^{2+}. Once washed, 5mL of prewarmed Tri-Reagent (Zymo Research, catalogue #R2050-1-200) was added, the sample was vortexed until homogenous, and placed in a 37ºC water bath for 5 minutes before being stored at -80ºC. The next day, the second culture was harvested and treated in the same manner as the first culture.

The samples were allowed to thaw before 1mL of chloroform was added, samples were shaken vigorously, and allowed to stand for two minutes at room temperature. After two minutes, the samples were spun down at 1400g for 30 minutes at 4ºC and the aqueous top layer supernatant collected. 2.5mL of 2-propanol was added to the collected supernatant, vortexed, then aliquoted to 1.5mL tubes and stored at 4ºC overnight. After the overnight precipitation, the tubes were spun down at 12,000g for 30 minutes at 4ºC, the supernatant decanted, and the pellets allowed to airdry. Once sufficiently dry, the pellets were treated with a DNase (Qiagen, catalogue #79254) per the manufacturer's protocol and combined with their respective samples before a standard sodium acetate precipitation was performed.

cDNA Synthesis and qPCR

cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalogue #4368814) normalized to the lowest RNA concentration. Additionally, a no reverse transcriptase control was included for both samples to assess the presence of contaminating genomic DNA.

qPCR data was generated using PowerTrack SYBR Green Master Mix (Applied Biosystems, catalogue #A46109) and run on QuantStudio™ 6 Flex by Vermont Integrative Genomics Resource's (VIGR) staff. Additionally, a no template control was included for each primer set used to assess for PCR contamination. Primers used for qPCR can be seen in Table 1 Supplemental One.

Data Handling and Visualization

The qPCR data were imported Microsoft Excel where data organization and analysis occurred. -ΔCt's equation was defined as elongation factor 1α GEO mean – the target gene's GEO mean. Data visualization was conducted in GraphPad Prism version 10.0.2 (GraphPad Software, San Diego, CA).

Results

Since there was no publicly available annotated genome or RNA-seq data for *Babesia duncani* at the time, qPCR primers were designed by identifying known highly expressed genes in other Apicomplexa then using National Center for Biotechnology Information (NCBI) protein Basic Local Alignment Search Tool (BLAST) to find the orthologous gene in *Babesia duncani*'s genome. *Elongation Factor-1α* (eF-1α) was chosen to normalize all other genes (-ΔCt) as it has been used extensively in other *Babesia* spp. transfection systems[6,7,8,9,10,11]. Additionally *eF-1α* is the only promoter to be described for *Babesia duncani*[12].

RT-qPCR data was generated from high parasitemia cultures (>30% parasitemia, data not shown) and is summarized in Figure 2 and Table 1. -ΔCt is the difference between the endogenous control gene, *eF-1α*, and the target gene with a positive value meaning the targe gene is more highly expressed than *eF-1α*. *Histone 2B* (H2B) had a mean -ΔCt for both timepoints of 2.25, indicating the *H2B* transcript was on average roughly 5-fold ($2^{2.25}$) more abundant than *eF-1α*'s transcript. The next highest mean -ΔCt promoters were genes such as *heat shock protein 70* (HSP70_Chr5), *HSP90*, and *lactate dehydrogenase* (LDH), which are involved in cellular stress responses. This was most likely due to the high parasitemia used to collect sufficient RNA. The last promoter with a positive -ΔCt was *enolase*, with a mean of 0.53 indicating on average roughly 1.5-fold ($2^{0.53}$) *enolase* transcript than *eF-1α* transcript.

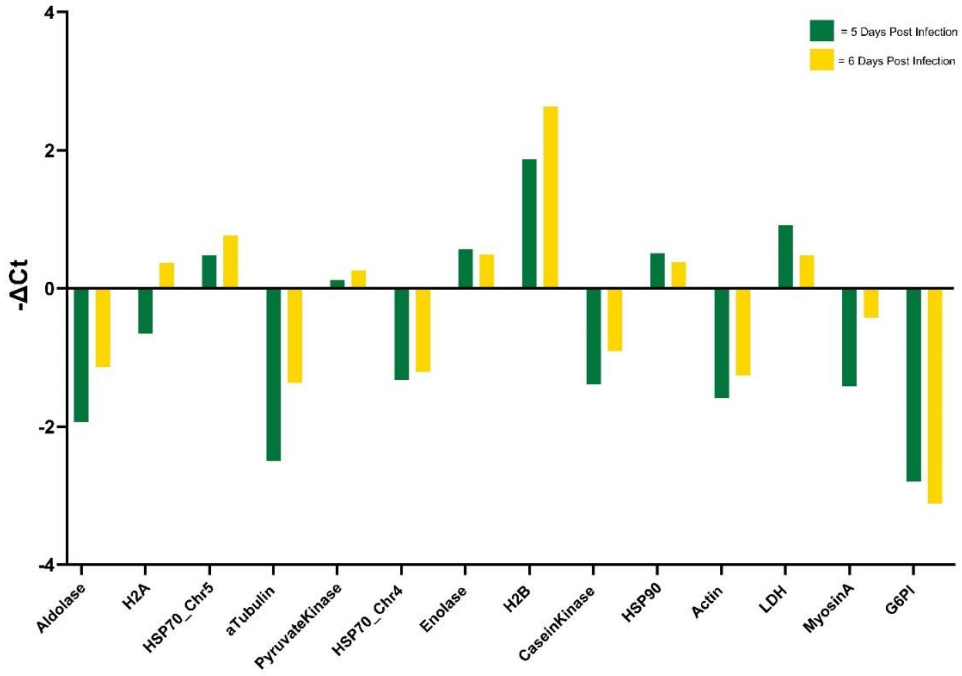

Figure 2. *Histone 2B* (H2B) is driven by a Strong Promoter. *H2B* had the strongest promoter of the genes tested with *enolase* being slighter stronger than *eF-1α*. Upregulation of *HSP70*, *HSP90*, and *LDH* is most likely due to high parasitemia as in a low parasitemia run these genes had lower -ΔCt values (data not shown). -ΔCt is the difference between the endogenous control gene, *eF1-α*, and the target gene with a positive value meaning the targe gene is more expressed than *eF1-α*. Abbreviations: *H2A = Histone 2A; HSP70_Chr5 = Heat Shock Protein 70 Chromosome 5; αTubulin = Alpha Tubulin; HSP70_Chr4 = Heat Shock Protein 70 Chromosome 4; H2B = Histone 2B; HSP90 = Heat Shock Protein 90; LDH = Lactate Dehydrogenase; G6PI = Glucose-6-Phopshate-Isomerase*. High parasitemia cultures were passed, allowed to grow, and collected at 5- or 6- days post infection. RNA was collected using the standard TRIzol-chloroform method, cDNA generated, and qPCR performed.

Table 1. Raw Cycle Thresholds Generated by qPCR. High parasitemia cultures were passed, allowed to grow, and collected at 5- or 6- days post infection. RNA was collected using the standard TRIzol-chloroform method, cDNA generated, and qPCR performed.

Gene	**Cycle Thresholds for 5 Days Post Infection Sample**	**Cycle Thresholds for 6 Days Post Infection Sample**
Aldolase	14.4	14.3
Histone 2A	13.1	12.8
Heat Shock Protein 70 Chromosome 5	12.0	12.4
Alpha Tubulin	14.9	14.5
Pyruvate Kinase	12.3	12.9
Heat Shock Protein 70 Chromosome 4	13.8	14.4

Enolase	11.9	12.7
Histone 2B	10.6	10.5
Casein Kinase	13.8	14.1
Heat Shock Protein 90	11.9	12.8
Actin	14.0	14.4
Lactate Dehydrogenase	11.5	12.7
Myosin A	13.9	13.6
Glucose-6-Phopshate-Isomerase	15.2	16.3
Elongation Factor-1α	12.4	13.2

Discussion

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) has been used previously to identify strong promoters of genes in other organisms[1,2,3]. Here, RT-qPCR has been used again to identify *Histone 2B*'s promoter (H2B) as the strongest promoter tested and *enolase*'s promoter as stronger than *elongation factor 1 alpha*'s (eF-1α) promoter. This indicates that *H2B* may be potentially suitable to drive constitutive expression of gene of interests in *Babesia duncani* transfections.

It is worth noting that the results of RT-qPCR may be influenced by primer efficiency. It was assumed that all primer sets used had the same primer efficiency instead of testing the efficiency, reacquiring primer sets, and retesting until they all had similar efficiency. While entirely unlikely, as the efficiency were untested, it is possible that *H2B* primers had efficiencies greater than 100% while the other genes' primers had efficiencies less than 100% thereby influencing the results.

One way to overcome this potential problem of primer efficiency, as well as any other confounding variables not identified, which was planned but never came to fruition, was testing the promoters *in vitro*. To do this, promoters, such as *H2B*, *eF1-α*, *actin* and *glucose-6-phophate-isomerase* (G6PI) would be used to drive a luciferase gene on identical plasmids. The luminescence would be compared of the plasmids with the expected results, based on the RT-qPCR data, being

H2B having the highest relative luminescence units (RLU), *eF1-α* the second highest RLU, *actin* the third, and *G6PI* the lowest. It is possible that transcriptional, translational, or post-transitional regulations and modifications cause unexpected results, which necessities this type of experiment in the future. This experiment would also solidify *H2B*'s promoter as suitable for driving constitutive expression of genes of interest in transfection for *Babesia duncani*.

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Chapter Two – Screening Transfection Protocols and Optimization

Introduction

Transfection is the introduction of foreign DNA into eukaryotic cells and can be achieved through a variety of means involving heat-shock, electrical-shock ("electroporation"), osmotic-shock, or fusion of lipid vesicle to the cells ("lipofection"). Not all transfections have the same efficiency, primarily due to their impact on cell viability and ability to deliver the DNA into cells. The efficiency of transfections is incredibly important, especially if a selectable marker or transient system is used but is often overlooked due to the headaches it causes. As such, most times researchers are able to get around low efficiency transfections and the need to optimize by increasing the amount of DNA used in the transfection and occasionally, increasing the number of cells transfected.

In the literature there is one published protocol for transfection of *Babesia duncani*, although the authors have noted that the efficiency of the transfection protocol is low[1] despite using a large number of cells and amount of DNA. Researchers have published optimized transfection protocols for other *Babesia spp*[2,3], with some even using the Lonza 4D Nucleofector, a machine available and used in the Huston laboratory for transfection of *Cryptosporidium*. Looking at *Plasmodium falciparum*, a related apicomplexan parasite that also infects RBCs, it has been shown *P. falciparum* can spontaneously pick up and express DNA that has been transfected into RBCs[4,5]. In a direct comparison of transfection methods of *P. falciparum*, the "indirect transfection" of DNA into RBCs that are later infected with *P. falciparum* outperformed "direct transfection" of *P. falciparum* infected RBCs using luciferase as an output[6].

The current study was conducted to establish the best transfection protocol from the published literature, based on transfection efficiency and overall health of the parasites following

transfection using a RT-qPCR readout. The best transfection protocol was then optimized by varying the number of parasites transfected and the amount of DNA used per transfection.

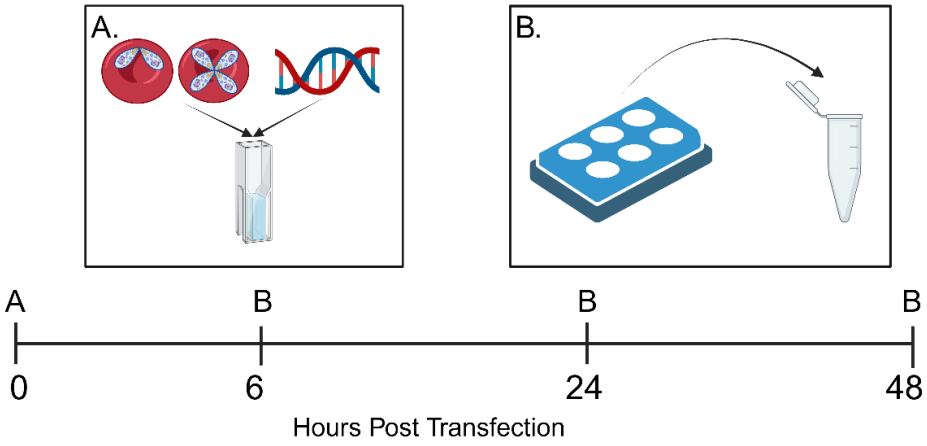

Figure 3. Timeline of Direct Transfection. A, At time 0-hours post transfection, infected RBCs (iRBCs) from a culture are collected, washed, counted, and aliquoted to a set number of Babesia per transfection. DNA is mixed with the aliquot of Babesia and the Babesia are transfected before being added to a previously prepared 5% hematocrit culture. B, At times 6-, 24-, and 48-hours post transfection, 50µL of culture was collected, flash frozen, and stored at -80ºC for later processing.

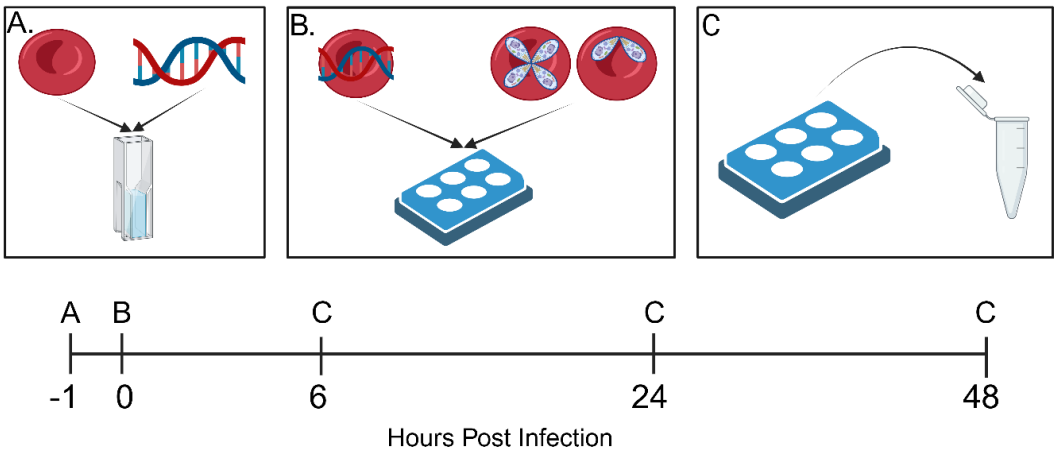

Figure 4. Timeline of Indirect Transfection. A, At time -1-pre transfection, uninfected RBCs (uRBCs) are washed, mixed with DNA, and transfected per their respective protocol. B, At time 0-post transfection, iRBCs from a culture are collected, washed, counted, and aliquoted to a set number of *Babesia* per transfection. The aliquot of Babesia mixed with the transfected uRBCs prepared as a 5% hematocrit culture. B, At times 6-, 24-, and 48-hours post transfection, 50µL of culture was collected, flash frozen, and stored at -80ºC for later processing.

Table 2. Transfection Protocols Screened, their Original Publications, and Tested Protocols Herein

Organism	Original Protocol	Publication	Tested Protocol
N/A	Lonza 4D Nucleofector, P3 Buffer, Program FP-158	Lonza Support, personal communication, January 12, 2022	As described in the original protocol
N/A	Lonza 4D Nucleofector, P3 Buffer, Program CM-150	Lonza Support, personal communication, January 12, 2022	As described in the original protocol
N/A	Lonza 4D Nucleofector, P3 Buffer, Program CM-138	Lonza Support, personal communication, January 12, 2022	As described in the original protocol
N/A	Lonza 4D Nucleofector, P3 Buffer, Program CM-113	Lonza Support, personal communication, January 12, 2022	As described in original protocol
Babesia duncani	BTX electroporator, settings 1,200 V, 25 µF, and pulsed twice	Wang et al, 2022[1]	Used a BTX ECM 630 with the settings 1,200 V, 25µF, 25Ω, and pulsed twice

Babesia gibsoni	Lonza 4D Nucleofector, SF Buffer, Program FA-113	Liu et al, 2017[2]	As described in the original protocol
Babesia microti	Lonza 4D Nucleofector, SF Buffer, Program FA-100	Liu et al., 2020[3]	As described in the original protocol
Plasmodium falciparum	Lyse-reseal	Govindarajalu et al, 2019[4]	As described in the original protocol
Plasmodium falciparum	Bio-Rad Gene Pulser, settings 0.31 kV and 960μF	Deitsch et al., 2001[5]	Used a BTX ECM 630 with the settings 1,200 V, 25μF, 25Ω, and pulsed twice

Methods

Culture Maintenance and Culture Preparation for Transfections

Culture maintenance was performed as previously described in Chapter One. To prepare cultures for transfections red blood cells were inoculated with *Babesia duncani* 3-4 days prior to transfection and the culture was allowed to develop into a vigorous infection. The day before transfection, parasitemia was checked by Giemsa stain to confirm parasitemia was ≥20%. On the day prior or day of transfection, fresh A+ blood was obtained.

On the day of transfection, an appropriate amount of blood for the number of transfections to be performed was washed three times with Puck's Saline Glucose + (PSG+). In a 24 well plate, 1mL 5% hematocrit cultures in cDMEM were made for each transfection with the washed blood before the plate was placed into a 37ºC, 5% CO_2 incubator until needed.

Plasmid Synthesis

The transient plasmid, GB4_NLuc, schematic used in this section is shown in Figure 1 Supplemental One. The parent plasmid's sequence, GB1_TT, was a kind gift from Dr. Lan He and was synthesized previously in the lab. GB4_nLuc was synthesized by digestion of GB1_TT with XhoI (New England Biolabs, catalogue #R0146S) and SpeI-HF (New England Biolabs, catalogue

#R3133S) followed by dephosphorylation using Antarctic phosphatase (New England Biolabs, catalogue #M0289S) and cleaned up with QIAquick PCR Purification Kit (Qiagen, catalogue #28104). Cleaved and dephosphorylated GB1_TT was used in GeneArt™ Gibson Assembly HiFi reaction (Invitrogen, catalogue # A46627) with synthesized oligonucleotide inserts from Integrated DNA Technologies per the manufacturer's protocol. Transformed bacteria were picked, plasmids screened, and eventually sequenced verified for the intended sequence to give GB4_nLuc.

To synthesize DNA for transfections, Sambrook & Russell, 2001[8] chloramphenicol amplification was used, and plasmid DNA was extracted using HiSpeed Plasmid Maxi Kit (Qiagen, catalogue #12662) per the manufacturer's protocol. Plasmid DNA was concentrated using a sodium acetate precipitation and stored at -20ºC until used.

Lonza Amaxa 4D Nucleofector Transfections

For nucleofection of infected red blood cells (iRBCs), the following protocol was used. Cells were washed twice with 1x phosphate buffer solution (PBS), no Ca^{2+} or Mg^{2+} and the pellet was resuspended in a final volume of 1mL with 1x PBS, no Ca^{2+} or Mg^{2+}. After the required number of parasites were aliquoted to a 1.5mL Eppendorf tube, the iRBCs were briefly spun down, and the supernatant removed. DNA was added and the final volume was brought up to 100µL with prechilled, completed P3 or SF buffer before the iRBCs were transferred to a cuvette. iRBCs were immediately nucleofected with the respective program before being transferred to the cultures prepared above.

BTX ECM 630 Electroporator Transfections

Wang et al, 2022[3] was followed with a few modifications. Briefly, a 0.2mm cuvette was pre-chilled on ice while cells were washed twice with 1x PBS, no Ca^{2+} or Mg^{2+} and the pellet was resuspended in a final volume of 1mL with 1x PBS, no Ca^{2+} or Mg^{2+}. After the required number of parasites were aliquoted to a 1.5mL Eppendorf tube, the iRBCs were briefly spun down, and the

supernatant removed before the pellet was washed with cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES (pH 7.6), 2 mM EGTA, and 5 mM $MgCl_2$). DNA was added and the final volume was brought up to 200µL with cytomix before the iRBCs were transferred to the prechilled cuvette. iRBCs were immediately electroporated using the BTX ECM 630 with the settings 1,200 V, 25µF, 25Ω, and pulsed twice before being transferred to the cultures prepared above.

For transfection of uninfected red blood cells (uRBCs), Deitsch et al., 2001[5] was followed with a few modifications. Briefly, a 0.4mm cuvette was pre-chilled on ice while 50µL of uRBCs was washed twice with 1x PBS, no Ca^{2+} or Mg^{2+} and once with cytomix. DNA was added and the final volume was brought up to 200µL with cytomix before the uRBCs were transferred to the prechilled cuvette. uRBCs were immediately electroporated using the BTX ECM 630 with the settings 1,200 V, 25Ω, 25µF, and pulsed twice before being transferred to a 1.5mL Eppendorf tube. The uRBCs were washed twice with pre-warmed cDMEM before brought up to 1mL in cDMEM, transferred to 24 well plate, and placed into a 37ºC, 5% CO_2 incubator until needed. iRBCs were then collected, washed, and counted as previously described. The required number of parasites were removed, spun down, resuspended in 50µL of cDMEM and used to infect the prepared culture.

Govindarajalu et al, 2019[4] Lyse-Reseal Transfections

For lyse-reseal transfections, Govindarajalu et al, 2019[4] was followed with some alterations. Briefly, 50µL of washed uRBCs was transferred to a 1.5mL Eppendorf tube, washed twice at 4ºC with ice-cold 1x PBS, no Ca^{2+} or Mg^{2+} then resuspended in 50µL of ice-cold lysis solution (5 mM K_2HPO_4, 1 mM ATP, pH 7.4) and DNA. The cells were incubated for 1 hour at 4ºC on a rocker before NaCl, $MgCl_2$, ATP, and Glutathione were added to 150 mM, 5 mM, 1 mM, and 1 mM respectively in a final volume of 110µL. After addition of the resealing reagents, the cells were incubated at 37ºC and shaken at 200 RPM for 1 hour. Once the hour was over, the cells were

transferred to a 15mL conical tube, washed twice with 10mL of pre-warmed cDMEM, before being resuspended in a final volume of 1mL in cDMEM, transferred to 24 well plate, and placed into a 37ºC, 5% CO_2 incubator until needed. iRBCs were then collected, washed, and counted as previously described. The required number of parasites were removed, spun down, resuspended in 50µL of cDMEM and used to infect the prepared culture.

Optimization of Transfection

To optimize the best transfection protocol, Govindarajalu et al, 2019[4] Lyse-Reseal, the number of parasites transfected, and amount of DNA used per transfection was varied. The previously described protocol was followed except the amount of DNA used was increased to 50ug from 30ug or decreased to 10ug, with the number of parasites transfected increased from $5x10^6$ to $5x10^7$ or decreased to $5x10^5$ in a separate set of experiments. The same controls, and readout were used with only a 6-hours post infection timepoint being analyzed.

Transfection Readout

50µL of culture from each condition was collected at 6-, 24-, and 48-hours post transfection, flash frozen in liquid nitrogen, and stored at -80ºC until it could be processed. Media was changed prior to collection of 6 and 24 hours post transfection time points. For the standard curve, 50µL of culture from >20% parasitemia culture was collected, flash frozen, and stored at -80ºC.

RNA from samples was extracted using RNeasy Mini Kit (Qiagen, catalogue #74104) with an on-column DNase digest per the manufacturer's protocol. cDNA was generated using Superscript III First-Strand Synthesis System (Invitrogen™, catalogue #18080051) with the random hexamer primers and 8µL from each sample per the manufacturer's protocol. In addition, a no reverse transcriptase control was included to assess the presence of contaminating plasmid and genomic DNA. qPCR data was generated as previously described except a standard curve method

was used. *Histone 2B* (H2B) was used as a proxy for parasitemia from a culture with a known parasitemia and plasmid DNA was used as a proxy for mRNA expression of the plasmid.

Data Handling and Visualization

The qPCR data were imported into Microsoft Excel where data organization and analysis occurred. Data visualization was conducted in GraphPad Prism version 10.0.2 (GraphPad Software, San Diego, CA).

Results

There is only one published transfection protocol for *Babesia duncani*. Therefore, we sought to test a variety of published transfection protocols in related *Babesia* spp. as well as *Plasmodium*. Despite several attempts with luciferase-encoding plasmids, including GB4_NLuc which encodes *nanoluciferase*, a more sensitive luciferase, no detectable signal was obtained in any of the protocols tested (data not shown). To assess whether this was due to lack of transfection efficacy or lack of sensitivity with the luciferase enzyme, RT-qPCR data was generated on one of the transfection attempts. The RT-qPCR targeted *nanoluciferase* present on GB4_NLuc and the data indicated that the *nanoluciferase* gene was being expressed (data not shown), indicating a sensitivity issue. Therefore RT-qPCR with a standard curve was chosen as the primary readout as it allowed examination of the transfection efficiency of various protocols as well as impact of transfection on *Babesia duncani* health.

Figure 5 shows the generated RT-qPCR data measuring parasitemia of the transfected cultures of the initial protocols screened, using Histone 2B (H2B) as a proxy. Govindarajalu et al, 2019[4] protocol had a steady growth in parasitemia across the timepoints and was the only protocol tested whose 48-hours timepoint parasitemia was higher than the 6-hours timepoint parasitemia. This steady increase in parasitemia is markedly different from the infected RBC (iRBC) control,

which exhibits a dip at the 24-hours timepoint before increasing again at the 48-hours timepoint. Additionally, Govindarajalu et al, 2019[4] had the third highest *nanoluciferase* transcript output in grams as seen in Figure 6, behind the protocol described in Wang et al., 2022[1] and the modified Dietsch et al., 2001[5] protocol. It is worth noting that despite use of filter pipet tips, bleaching of workspace and pipettes, DNase treatment, and other good work practices the high background observed in iRBC and uRBC is due to environmental contamination of the GB4_NLuc plasmid.

After all the protocols were screened, we sought to choose one for optimization based on four criteria: 1. General ease of the protocol; 2. Ability to transfect a large number of cells at once; 3. Impact of the protocol on *Babesia duncani*'s ability to grow following transfection and; 4. Ability of the protocol to get DNA into *Babesia duncani*. Using these four criteria, Govindarajalu et al, 2019[4] lyse-reseal protocol was chosen as it technically simple to perform, only requires common laboratory reagents, is able to transfect a large number of cells at once as it relies on spontaneous uptake of DNA from reseal RBCs, allowed *Babesia duncani* to growth after transfection as seen in Figure 5, and Figure 6 shows it had the third highest *nanoluciferase* transcript output of the test protocols.

To optimize the lyse-reseal protocol, the amount of *Babesia duncani* infected cells used per transfections was varied as well as the amount of DNA used in the lyse-reseal protocol. Figure 7 shows the generated RT-qPCR data measuring parasitemia and nanoluciferase of transfected cultures. Panel A shows percent parasitemia, which uses *Histone 2B* (H2B) as a proxy. As expected, infecting with more parasites gives a higher parasitemia at the 6-hours timepoint, seen in the $5x10^7$ parasites sample, while infecting with less parasites gives a lower and lower parasitemia at the same timepoint, seen in the standard (which used $5x10^6$ parasites), and $5x10^5$ samples. Additionally, the amount of DNA loaded into the RBCs did not seem to have a significant impact on parasitemia as the 10µg, standard (which loaded 30µg), and 50µg DNA loaded samples all had comparable

parasitemia at the 6-hours timepoint. Interestingly, despite being infected with the same number of parasites, $5x10^6$, the iRBC control had a noticeably lower parasitemia than the 10µg, standard, and 50µg DNA loaded samples. Panel B shows the amount of *nanoluciferase* transcript output in grams. Predictably, but admittedly not as substantial as expected, increasing the amount of DNA loaded into the RBCs increases the amount of *nanoluciferase* transcript output, seen with the incremental increase of *nanoluciferase* transcript from 10µg to standard (30µg) to 50µg of DNA. Also predictably, infecting with more parasites gives more *nanoluciferase* transcript, seen in the $5x10^7$ parasites sample, while infecting with less parasites gives a lower and lower amount of *nanoluciferase* transcript, seen in the standard ($5x10^6$ parasites), and $5x10^5$ samples. Interestingly, for both the DNA and number of parasites used, the difference between the highest amounts used (50µg and $5x10^7$) and the standard (30µg and $5x10^6$) is not in the same magnitude as the difference between the standard and the lowest amounts used (10ug and $5x10^5$). This indicates there is some limit to the efficiency of this protocol that fundamentally cannot be overcome without modifying it.

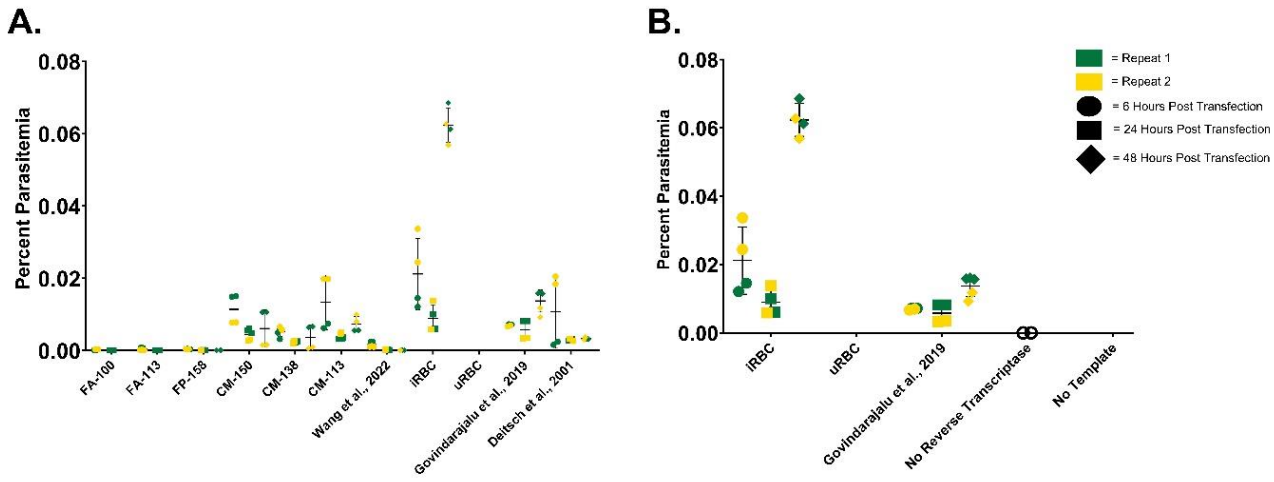

Figure 5. Govindarajalu et al, 2019[4] Lyse-Reseal Protocol Gives a Small but Appreciable Growth in Parasitemia Following Transfection. A, The Lyse-Reseal Protocol was the only protocol tested that had a 48-hours timepoint where the parasitemia was higher than the initial 6-hours timepoint. Other transfection protocols, such as Lonza Amaxa 4D

Nucleofector's CM-150, CM-138, and CM-113 had higher parasitemia at the 48-hours timepoint compared to the 24-hours timepoint but these 48-hours timepoints were less than the 6-hours timepoint. Interestingly, the results here echo what Wang et al., 2022[3] observed with their protocol, being a huge decrease in the parasitemia following transfection. B, The Lyse-Reseal Protocol timepoints with the experimental control timepoints and RT-qPCR controls. Samples were collected 6-, 24-, and 48-hours post transfections and stored at -80ºC until RNA could be extracted, cDNA synthesized, and qPCR data generated. Histone 2B (H2B) was used as a proxy for parasitemia with a standard curve used from a culture with a known parasitemia.

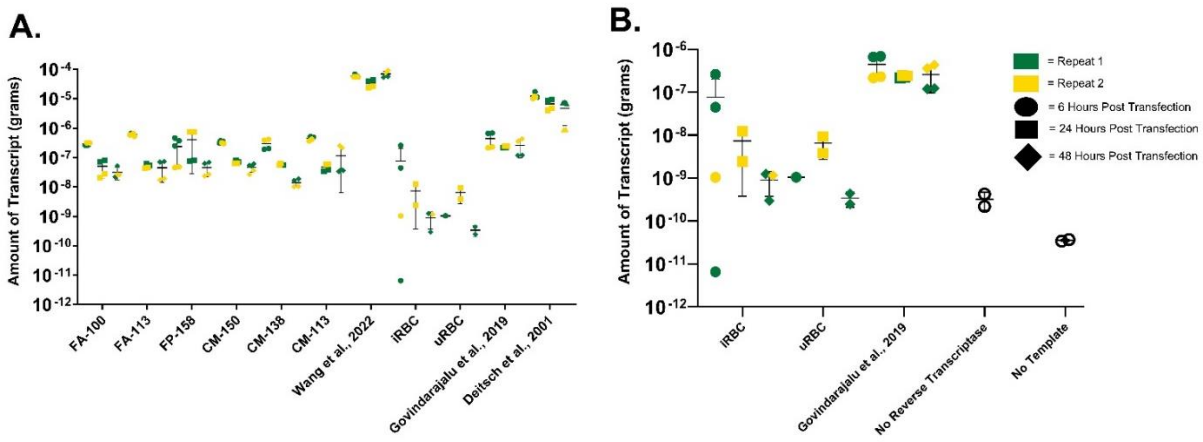

Figure 6. Govindarajalu et al, 2019[4] Lyse-Reseal Protocol gives Modest *Nanoluciferase* Expression. A, The Lyse-Reseal Protocol gave the third highest transfection efficiency measured as amount of *nanoluciferase* transcript, behind Wang et al., 2022[1] and Dietsch et al., 2001[5] modified protocols. These protocols produced comparable levels of *nanoluciferase* transcript across the timepoints within their respective protocols. The other protocols tested produced some *nanoluciferase* transcript at the 6-hours timepoint that quickly fell off at later timepoints. B, The Lyse-Reseal Protocol timepoints with the experimental control timepoints and RT-qPCR controls. It is likely that despite use of filter pipet tips, bleaching of workspace and pipettes, DNase treatment, and other good work practices the high background observed in iRBC and uRBC is due to environmental contamination of the GB4_NLuc plasmid. Samples were collected 6-, 24-, and 48-hours post transfections and stored at -80ºC until RNA could be extracted, cDNA synthesized, and qPCR data generated. GB4_NLuc plasmid was used as a proxy for the amount of *nanoluciferase* transcript with a standard curve used from a known amount of plasmid in grams.

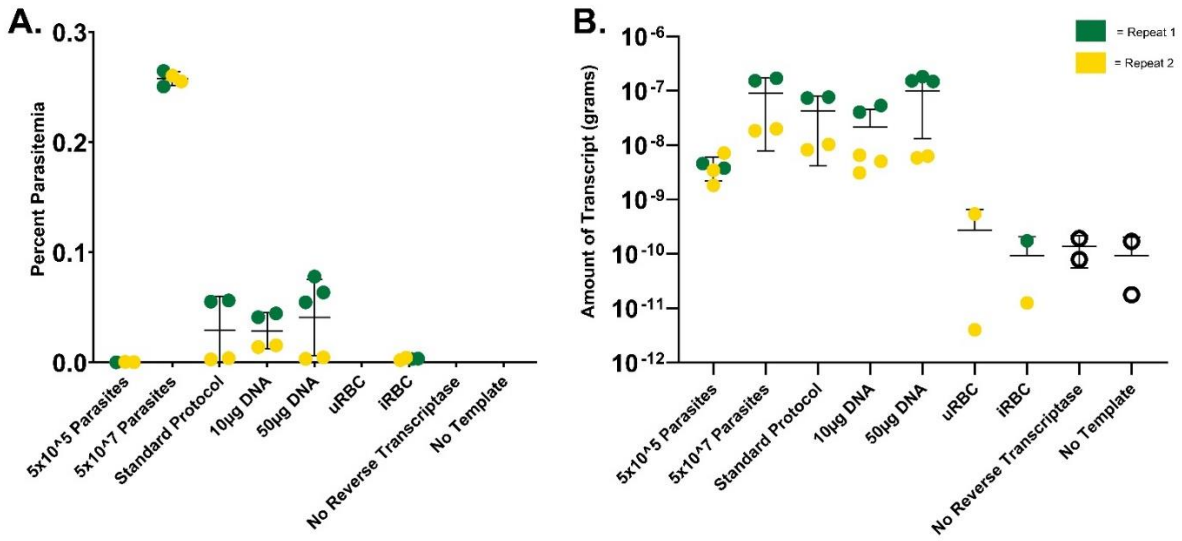

Figure 7. Varying of Total DNA Input and Starting Parasite Numbers into Govindarajalu et al, 2019[4] Lyse-Reseal Protocol Reveals Increasing the Starting Parasite Numbers is Most Impactful for Transfection Efficiency. A, Infecting with more parasites gives higher parasitemia, seen with the $5x10^7$ parasites sample, while infecting with less parasites gives a lower parasitemia, seen in the standard (which used $5x10^6$ parasites), and $5x10^5$ parasites samples. The amount of DNA loaded into the RBCs did not seem to impact parasitemia as the 10µg, standard (which loaded 30µg), and 50µg DNA loaded RBC samples all had comparable parasitemia. Interestingly, despite being infected with the same number of parasites, $5x10^6$, the iRBC control had a noticeably lower parasitemia than the 10µg, standard, and 50µg DNA loaded samples. B, Increasing the amount of DNA loaded into the RBCs increases the amount of *nanoluciferase* transcript output, seen with the incremental increase of *nanoluciferase* transcript from 10µg to standard (30µg) to 50µg of DNA. Also infecting with more parasites gives more *nanoluciferase* transcript, seen in the $5x10^7$ parasites sample, while infecting with less parasites gives a lower amount of *nanoluciferase* transcript, seen in the standard ($5x10^6$ parasites), and $5x10^5$ parasites samples. Samples were collected 6-hours post transfection and stored at -80ºC until RNA could be extracted, cDNA synthesized, and qPCR data generated. Histone 2B (H2B) was used as a proxy for parasitemia with a standard curve used from a culture with a known parasitemia and GB4_NLuc plasmid was used as a proxy for the amount of *nanoluciferase* transcript with a standard curve used from a known amount of plasmid in grams.

Discussion

In the literature it is noted[1,9] that a reliable transfection method is desperately needed for *Babesia duncani* to facilitate studying of the parasite's biology and molecular genetics. Herein, several different transfection protocols from the *Babesia* and *Plasmodium* literature were screened for their efficiency, not only getting DNA into *Babesia duncani* and expression but also on cell viability. From the initial screen, Govindarajalu et al, 2019[4] Lyse-Reseal protocol showed modest expression of the *nanoluciferase* gene from the plasmid while allowing the parasites to grow. Other protocols showed high *nanoluciferase* expression but killed the parasites, such as Wang et al 2022[1],

little *nanoluciferase* expression and killed the parasites, such as FP-158, and little *nanoluciferase* expression without killing the parasites, such as CM-113. To optimize the Govindarajalu et al, 2019[4] Lyse-Reseal protocol, the number of parasites used to infect the DNA loaded RBCs was varied from the standard $5x10^6$ and the amount of DNA loaded into the RBCs was varied from the standard 30µg. Interestingly, despite being infected with the same number of parasites, $5x10^6$, the DNA varied, and standard experimental condition had markedly higher parasitemia than the iRBC. This may indicate that the lyse-resealed RBCs are preferentially or more easily invaded by *Babesia duncani*. Not surprisingly, using more parasites and more DNA per transfection gave more *nanoluciferase* expression, although there appears to be a limitation to the amount of transfection efficiency that can be gleaned from increasing the number of parasites and amount of DNA. This limitation can be observed in the diminishing return of *nanoluciferase* expression with both increasing the number of parasites and the amount of DNA used.

There are two potential ways to overcome this inherent limitation. The first way is to use another lyse-reseal protocol to get the DNA into the RBCs. As there are several lyse-reseal protocols reported in the literature, it would be best to test several different ones with the following simple experimental protocol. A set amount of DNA would be used for the different RBC lyse-reseal protocols then once the RBCs are resealed, the cells would be stained with a cell-permeable nucleic acid stain, such as Hoechst 33528. The samples would then be run through flow cytometry to identify what percentage of cells are carrying DNA, with the highest percentage of DNA carrying RBCs being the best protocol. Three lyse-reseal protocols are presented that could be compared as a starting point, including the one utilized in this work. Govindarajalu et al, 2019[4] does not touch on the efficiency of getting DNA into the RBCs undergoing the lyse-reseal protocol. The authors touch on the percentage of RBC lysate that is resealed without providing a methodology for how this is measured and show how little DNA they can use to get a reliable transfection. Their protocol

is based on Murphy et al., 2006[10] lyse-reseal protocol in which the authors showed with FITC-labeled dextran that their protocol gave a >99% population of lyse-resealed RBCs containing the FITC-labeled dextran. Dluzewsk et al., 1983[11] was the first paper published about the lyse-reseal method but does not touch on its efficiency.

The second way to way to overcome the inherent limitation of the lyse-reseal protocol is to develop a method of lifecycle synchronization for *Babesia*. Currently there is no reported successful attempts to synchronize *Babesia* cultures in a manner akin to *Plasmodium*. This matters because it is believed only one life stage of *Plasmodium*, the trophozoite, can spontaneously pick up DNA shortly after invasion of the RBC[4,5]. The synchronization methods used by *Plasmodium* researchers[12,13,14,15] allow the researchers to tightly control the life stages of their cultures and maximize the chance of a successful transfection while using the lyse-reseal protocol. It should be noted prior to this work, the following have been attempted in this lab for their ability to synchronize *Babesia* culture: Guanidine hydrochloride[13], glucose[13], glycine[13], a Ficoll gradient[16], incubation at 4ºC[17], and passage through a 1.2µm filter twice[18].

While the background of the amount of *nanoluciferase* transcript may seem high, this is more than likely due to environmental contamination of the samples with the GB4_NLuc plasmid due its inconsistent nature. Precautions, such as bleaching the workspace and pipettes before each use, using filter pipette tips, using an unopened DEPC-treated water, and treating RNA with DNase during extraction, were taken to minimize contamination with GB4_NLuc plasmid. Together with the inherent limitation of the presented lyse-reseal protocol, it may be tempting to look at other transfection protocols with promising results presented herein, such as Wang et al., 2022[1], as it had the highest amount of *nanoluciferase* transcript, but this is not advisable. This is because the results presented herein recapitulate what has been reported in the literature for *Plasmodium* and *Babesia*. In “Comparison of the absolute and relative efficiencies of electroporation-based transfection

protocols for *Plasmodium falciparum*" by Hasenkamp et al., 2012, it was found that the DNA loaded erythrocyte method is nearly 200 times more efficient than direct transfection of iRBCs. Not only were the efficiency much greater but successful transfected parasites were recovered nearly twice as fast in a workable state (>1% parasitemia) which is seen in the work herein. For *Babesia*, DNA loaded RBCs has been shown to provide recovery of successful transfection faster than direct transfection of iRBCs or free merozoites in *Babesia divergens*[19]. The work in *Babesia divergens*[19] was unknown to this work herein, strengthening the claims of both works and making this the second work to show spontaneous uptake of DNA by *Babesia* and first for *Babesia duncani*.

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Chapter Three – Development of a CRISPR/Cas9 System in *Babesia duncani*

Introduction

Recently classical genetic modification techniques have been described for *Babesia duncani*[1]. These techniques rely on maintenance of a plasmid with a selectable marker by *Babesia duncani* and homologous recombination of a linearized fragment with a selectable marker[1]. However, these techniques have several limitations namely, required maintenance of plasmid, low efficiency of genome integration paired with the long time usually required for drug selection, and inability to manipulate multiple genes in a single parasite using a single technique.

In other *Babesia* species, *Babesia bovis*[2], and other Apicomplexa parasites, *Cryptosporidium*[3], *Plasmodium*[4], and *Toxoplasma*[5], precise and site-specific genome editing techniques are available through Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 endonuclease mediated systems. CRISPR/Cas9 works by producing a site-specific double strand break in the target DNA through Cas9's nuclease activity. Cas9 is guided to the target DNA by a single guide RNA (gRNA) which is customized to the template. The double strand break is then repaired through homologous recombination, as *Babesia* spp. lack non-homologous end joining[6], which can be used to insert desired sequences into the target DNA.

The current study was conducted to develop a CRISPR/Cas9 system for *Babesia duncani* by demonstrating a targeted knock-in of a mCherry-human dihydrofolate reductase (hDHFR) fusion into *Babesia duncani*'s *thioredoxin-1* gene locus.

Methods

Culture Maintenance and Culture Preparation for Transfections

Culture maintenance and preparation for transfection was performed as previously described in Chapter One and Chapter Two.

Plasmid Synthesis

The CRISPR-Cas9 plasmid, TPXgRNA_GB3_CC9, and repair cassette, TPX_FsdRC_GB3, used in this section are shown in Figure 1 Supplemental One. TPXgRNA_GB3_CC9 was created by insertion of a guide RNA into two artificial, inverted PaqCI (New England Biolabs, catalogue #R0745) sites in downstream *Babesia duncani*'s U6 promoter and upstream the trans-activating CRISPR RNA (tracrRNA) consensus sequence in GB3_CC9. GB3_CC9 and TPX_FsdRC_GB3 were both synthesized by digestion of pUC19 with HincII (New England Biolabs, catalogue #R0103) followed by dephosphorylation using Antarctic phosphatase (New England Biolabs, catalogue #M0289S) and cleaned up with QIAquick PCR Purification Kit (Qiagen, catalogue #28104). Cleaved and dephosphorylated pUC19 was used in GeneArt™ Gibson Assembly HiFi reaction (Invitrogen, catalogue # A46627) with synthesized oligonucleotide inserts from Integrated DNA Technologies per the manufacturers protocol. Transformed bacteria were picked, plasmids screened, and eventually sequenced verified for the intended sequence to give GB3_CC9 and TPX_FsdRC_GB3 respectively.

To synthesize DNA for transfections, TPXgRNA_GB3_CC9 plasmid DNA was extracted using Pureyield™ Plasmid Midiprep System (Promega, catalogue #A2492) per the manufacturer's protocol. TPX_FsdRC_GB3 plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, catalogue #27104) per the manufacturer's protocol. Extracted TPX_FsdRC_GB3 plasmid was then used in a polymerase chain reaction (PCR) with TPX_FsdRC_GB3_Lin_For, TPX_FsdRC_GB3_Lin_Rev and Phusion U Green Hot Start DNA Polymerase (Thermo Scientific,

catalogue #F556) per the manufacturer's protocol to generate linearized TPX_FsdRC_GB3 with the PCR product cleaned up with QIAquick PCR Purification Kit (Qiagen, catalogue #28104). All generated DNA was concentrated using a sodium acetate precipitation and stored at -20ºC until used.

TPXgRNA with Repair Cassette Transfection

Transfections were performed using the modified Govindarajalu et al, 2019[7] Lyse-Reseal as previously described. 50µg of TPXgRNA_GB3_CC9 and 50µg of linearized TPX_FsdRC_GB3 was used with the transfected red blood cells (RBCs) infected with $5x10^6$ parasites. An infected RBC (iRBC), uninfected RBC (uRBC), and iRBC treated with WR99210 were included as controls.

To select for successful CRISPR-edited parasites, 5 nM WR99210 (Sigma, catalogue #SML2976) was added to the media after 24 hours post transfection. WR99210 dosage was increased to 20 nM at 10 days post transfection.

TPXgRNA without Repair Cassette Transfection and PCR

Transfections were performed using the modified Govindarajalu et al, 2019[7] Lyse-Reseal as previously described. 50µg of TPXgRNA_GB3_CC9 was used with the transfected red blood cells (RBCs) infected with $5x10^6$ parasites. Three timepoints, 3-, 8-, and 24-hours post transfection, cultures were set up with iRBC and uRBC controls.

RNA from cultures was extracted using the Trizol-Chloroform RNA extraction as previously described. At the various timepoints, the entire culture was harvested and stored until all samples were collected. iRBC and uRBC controls were harvested alongside the 24-hour post transfection culture. cDNA was generated using Superscript III First-Strand Synthesis System (Invitrogen™, catalogue #18080051) with the random hexamer primers and 8µL from each sample

per the manufacturer's protocol. In addition, a no reverse transcriptase control, using the 3-hour timepoint, was included to assess the presence of genomic DNA.

5µL of generated cDNA was used for PCR targeting TPXgRNA_GB3_CC9 and Histone 2B (H2B). H2B PCR product was generated using Phusion U Green Hot Start DNA Polymerase (Thermo Scientific, catalogue #F556) per the manufacturer's protocol. TPXgRNA_GB3_CC9 PCR product was generated using Platinum^{TM} Taq DNA Polymerase (Invitrogen, catalogue #15966005) per the manufacturer's protocol. PCR products were run on a 1% agarose gel with ethidium bromide in 1x Tris-Borate-EDTA (TBE) with a Generuler 1kb DNA ladder (Thermo Scientific, catalogue #SM0311) for reference.

Cell Staining, Imaging, and Image Processing

Cells were stained with 20 µM Hoescht 33528 in culture media for 10 minutes before being washed twice with 1x phosphate buffer solution, no Ca2+ or Mg2+ (1x PBS). Cells were then resuspended in 1x PBS and imaged with a Nikon widefield epifluorescence microscope equipped with a 1.4-megapixel EXi blue CCD camera at 40x dry (NA = 0.75) with the appropriate controls.

Z-stacks were exported to AutoQuant X3 (Meyer Instruments) and deconvoluted using the default settings. Deconvoluted images were then exported to ImageJ, combined into maximum intensity projections, pseudo-colored, and overlayed.

Results

As the first attempt at CRISPR/Cas9 in *Babesia duncani*, there were several things that could go wrong and require troubleshooting. Therefore, to help validate any mCherry expressing *Babesia duncani* observed by microscopy post transfection, the expression of the *S. pyogenes* Cas9 (SpCas9) encoded on TPXgRNA_GB3_CC9 was checked by reverse transcriptase (RT)-PCR. This

was done because when TPXgRNA_GB3_CC9 was designed and constructed, the results of Chapter One had not been generated so *actin* was chosen to drive SpCas9 expression, which is now known to not be a strong promoter compared to *elongation factor-1α* (eF-1α). Additionally, SpCas9 was codon optimized using *Babesia bovis* codon usage as there was, and still is, no available codon usage table for *Babesia duncani*. Therefore, we thought it prudent to check if SpCas9 was expressed or not following transfection.

To assess if SpCas9 on TPXgRNA_GB3_CC9 was expressed or not, RNA from transfected cultures was collected, cDNA generated, and PCR targeting SpCas9 performed. Figure 8 shows that SpCas9 is indeed expressed following transfection, panel A, while in the presence of a *Babesia duncani* marker (*histone 2B* (H2B)), panel B, at the same timepoint. The lack of any bands in the no reverse transcriptase lanes indicates that the cDNA synthesis and PCR was performed on pure RNA, further solidifying that SpCas9 encoded on TPXgRNA_GB3_CC9 is expressed in *Babesia duncani*. This means that any mCherry transgenic parasites observed by microscopy are potentially real.

With the knowledge that a SpCas9 encoded on TPXgRNA_GB3_CC9 was expressed, a transfection was TPXgRNA_GB3_CC9 and linearized TPX_FsdRC_GB3 was performed. Transfected RBCs were monitored for potential transgenic, mCherry expressing *Babesia duncani* by microscopy such as the one presented in Figure 9. The presented images are a single Z-stack that has been deconvoluted (AutoQuant X3, Meyer Instruments), combined into maximum intensity images (ImageJ), and overlayed with each other. Non-specific staining or fluorescent signal was not observed in the controls at the same exposure times (data not shown).

The transfected culture was kept under 20 nM WR99210 drug pressure, increased from 5 nM on day 10 post transfection, for roughly two months and check regularly for an increasing number of mCherry expressing *Babesia duncani.* The non-transfected WR99210 pressured infected

control had no observable parasites by day 13 and was discarded on day 25, along with the non-transfected infected control and non-transfected uninfected control. However, no other believable (not autoflourescent) mCherry expressing *Babesia duncani* were observed after the one present in Figure 9.

Figure 8. *S. pyogenes Cas9* (SpCas9) encoded on TPXgRNA_GB3_CC9 is expressed by Babesia duncani. A, PCR targeting SpCas9 on generated cDNA shows SpCas9 is expressed at all timepoints RNA was collected. Expression appears to peak at the 8-hours timepoint before dropping slightly at the 24-hours timepoint, although this is difficult to say based solely on the intensity of the bands. B, PCR targeting *histone 2B* (H2B) shows parasites are present at the timepoints RNA and SpCas9 expression was seen. Interestingly, no amplicon was seen in the iRBC lane which would be expected. This may be due to the subpar condition of isolated RNA as it had carried over Trizol indicated by a peak at 270nm on the nanodrop reading. Samples were collected at 3-, 8-, and 24-hours post transfection and stored at -80ºC until RNA could be extracted, cDNA synthesized, PCRs and gels run. TPXgRNA_GB3_CC9's positive PCR control was diluted plasmid and H2B positive PCR control was previously generated Babesia babesia iRBC lyse-reseal cDNA.

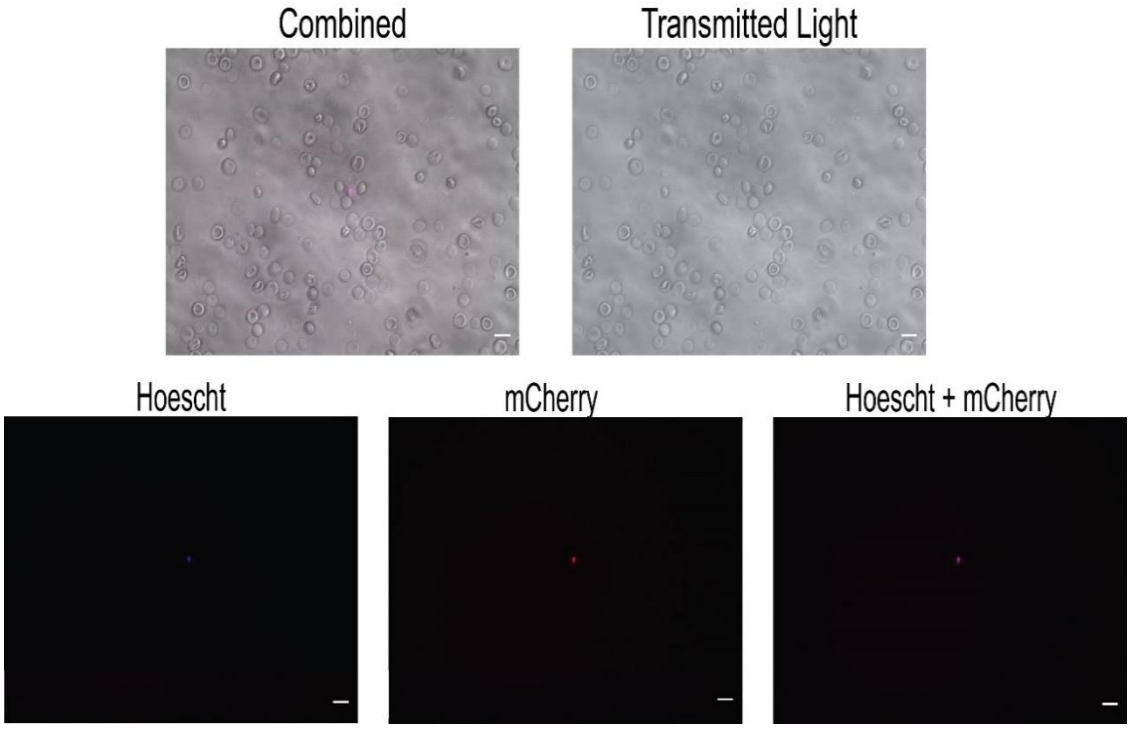

Figure 9. Potential mCherry Expressing Babesia duncani due to CRISPR/Cas9 editing 24-hours Post Transfection. 24-hours post transfection with TPXgRNA_GB3_CC9 and linearized TPX_FsdRC_GB3, media was changed, and cells were taken for imaging. Cells were stained with 20 µM Hoechst 33528 for 10 minutes before being washed twice with 1x PBS. Cells were resuspended in 1x PBS and imaged with a Nikon widefield epifluorescence microscope equipped with a 1.4-megapixel EXi blue CCD camera at 40x dry (NA = 0.75) with the appropriate controls (data not shown). Scale bars are 10 µm long. Images presented are a single Z-stack that have been deconvoluted (AutoQuant X3, Meyer Instruments) and presented as maximum intensity projections with pseudo-coloring (ImageJ). Pseudo-Coloring: Blue = Hoechst 33528; Red = mCherry

Discussion

Currently there is no published report describing Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 system in *Babesia duncani*. Herein is the first potentially successful CRISPR/Cas9 system in *Babesia duncani*. From pure RNA, cDNA was generated and shows that *S. pyogenes* Cas9 (SpCas9), encoded on TPXgRNA_GB3_CC9, expressed by *Babesia duncani*. Additionally, a potentially mCherry expressing *Babesia duncani*, which could only occur by CRISPR/Cas9 editing, was observed by microscopy 24-hours post transfection.

These are promising results, indicative that the CRISPR/Cas9 system is working in its current form, however as it stands this work is incomplete. To complete this work, more mCherry

expressing parasites need to be observed and integration of the repair cassette needs to be verified. Despite being under drug pressure for more than a month, no other believable mCherry expressing parasites have been observed since the 24-hours post transfection one. Verification of integration of the repair cassette can easily be accomplished once a culture of transgenic parasites have been dilution cloned. To verify the integration of a repair cassette, genomic DNA would be isolated and PCR would be performed over the gaps where integration should have occurred.

For future attempts of CRISPR/Cas9 in *Babesia duncani*, there are several improvements that could be made. The easiest would be to use the optimized transfection protocol parameter generated in this work; namely infecting the lyse-resealed RBCs with more parasites. The ratio of CRISPR/Cas9 plasmid to repair caste could be changed from 1:1 to 1:5, ensuring if *SpCas9* does cut the genome a repair cassette is nearby. This is the standard ratio used in *Toxoplasma*[5] and has been used in *Plasmodium*[4,20]. Another easy improvement would be to slowly increase the drug selection as all reported WR99210/hDHFR system in *Babesia* have been under the *elongation factor-1α* promoter[1,2], not the *actin* promoter. This may necessitate a slow increase in selection due to *actin*'s lackluster promoter and allowing accumulation of hDHFR enzyme. A variation of this would be to delay the selection of transgenic parasites, which has seen success in *Plasmodium* CRISPR/Cas9 transfection systems[20]. It should be cautioned however that if the resulting transgenic parasite has any fitness cost due to the CRISPR/Cas9 editing, delaying selection may not be ideal. Other improvements include those discussed in Chapter Two's discussion regarding the inherent limitation of the lyse-reseal protocol, redesigning TPXgRNA_GB3_CC9, redesigning TPX_FsdRC_GB3, and redesigning the integration site of the repair cassette. When redesigning TPXgRNA_GB3_CC9, a stronger promoter should be used to drive the expression of SpCas9 than *actin*. TPXgRNA_GB3_CC9 was synthesized before the results of Chapter One had been generated and *actin* is highly expressed in other apicomplexans, even in some *Babesia* spp[8,9]. In addition,

SpCas9 should have its codon optimized for *Babesia duncani* as it is codon optimized for *Babesia bovis*, although this may prove more difficult than it is worth as there is no curated list of annotated genes for *Babesia duncani*. For redesigning TPX_FsdRC_GB3, like TPXgRNA_GB3_CC9 it was synthesized before the promoter RT-qPCR data was generated, therefore a stronger promoter should be chosen to drive the repair cassette. Additionally, a different fluorescent protein may be considered[10] but the farther into the light spectrum is suggested as hemoglobin is well document to absorb spectrum below >600nm[11]. Lastly, the ambiguous nature of *thioredoxin-1* locus in *Babesia duncani* genome may require redesigning of the integration site of the repair cassette. Currently there are two published "annotated" genomes[12,13] for *Babesia duncani*, however they are both unfinished on NCBI and differ on several important elements; number of chromosomes, location of genes, location of exons and introns, number of genes encoded. This can make a clean knock-in, such as the one describe herein, difficult to attempt and be successful. It could make an unclean knock-in of *thioredoxin-1* that would produce an unviable transgenic in the long-run as the linearized TPX_FsdRC_GB3 repair cassette does not encode a 3' non-coding region. This is problematic as it may cause the repair cassette mRNA to be rapidly degraded before it can be translated into protein. The current TPXgRNA_GB3_CC9 targets the locus of *thioredoxin-1* given by Wang et., al, 2022[11] as it was the only available data when TPXgRNA_GB3_CC9 was synthesized.

Before looking ahead at the nearly endless possibilities for a working CRISPR/Cas9 system in *Babesia duncani*, it would be wise to look at the needed immediate genetic tools. These genetic tools are multiple developed manipulatable gene loci and selectable markers. *Hook associated protein-2* (HAP-2) and *6-Cys-E* have been shown to be dispensable in *Babesia bovis*[14,15] and would open the pathway for tick-stage work. *Rad51* and *elongation factor-1α* have also been shown to be dispensable in *Babesia bovis*[16,17]. Blasticidin and puromycin have been used as selectable for

Babesia bovis[17] and *Babesia gibsoni*[18] respectively but *Babesia duncani* is reportedly uninhibited by blasticidin[1]. *Cryptosporidium* has only had paromomycin as a selectable marker but recently they gained a lysyl-tRNA synthetase selectable marker that should also be explored[19].

Now actually looking ahead to when the CRISPR/Cas9 system is working for *Babesia duncani* there are nearly endless possibilities for its use. As there are nearly endless possibilities, a brief list of the main broad categories will be presented. They include knockout of genes to explore gene functions and host-pathogen interaction; creation of reporter parasites for *in vivo* and *in vitro* assays or needs; drug target identification validation; creation of attenuated strains for vaccine or immunotherapy purposes. CRISPR/Cas9 has been successfully used for these purposes in other apicomplexans and will surely be used for these purposes once a CRISPR/Cas9 system is successfully working in *Babesia duncani*.

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Chapter Four - 384-Well High Throughput Compound Screening Format for *Babesia duncani*

Introduction

High throughput compound screening (HTS) is a discovery process that allows fast, procedurally simple, and cost-effective testing of an astronomical number of compounds against some biological target. The biological target in question can be an organism, such as a parasite, a specific cell type, such as cancerous cells, or enzymes. Regardless of the biological target, a robust HTS allows for identification of "lead" compounds that can undergo optimizations to create more potent compounds. Once a handful of optimized compounds are synthesized, they can enter the drug pipeline where important pharmacokinetic and pharmacodynamic information, such as toxicity, bioavailability, and mechanism of action, can be gathered. Additionally, while most compounds fail to make it through the drug pipeline due to toxicity issues or poor *in vivo* efficacy, many can find new use as chemical probes of biological processes. As identified inhibitors, these compounds offer potential advantages over genetic manipulations as they can be temporally controlled, are easier to work with, and are less likely to induce compensatory gene changes.

Currently, the drug pipeline for *Babesia duncani* is slow and tedious despite a rising need for effective treatments against it and other causes of human babesiosis[1,2,3,4,5]. The slow and tedious nature can be owned up to the "high" throughput compound screening method being used to screen compounds. That method utilizes a 96-well plate format[6,7,8,9] which severely limits the number of compounds that can be screened compared to a 384-well plate, among other things. Other Apicomplexa parasites, such as *Cryptosporidium*[10], *Plasmodium*[11], Toxoplasma[12], *Eimeria*[13], and *Cystoisospora*[14], have *in vitro*, cell based 384-well HTS assays. This allows for fast identification of lead compounds from compound libraries and testing of those optimized compounds, leading to an overall faster and seamless drug pipeline than is seen with *Babesia duncani*.

The current study was conducted to develop a 384-well HTS format for *Babesia duncani*, validate the HTS method using an established statistical metric for HTS known as the Z' score, and test compounds to identify potential leads for further optimization.

Methods

Culture Maintenance and Culture Preparation for Compound Screening

Culture maintenance was performed as previously described in Chapter One. For compound screening, fresh A+ blood was obtained every week, and the same lot of blood was used for the compound testing as the parasites were cultured in. Four days before the start of a compound testing, a 10mL, 5% hematocrit culture was infected to 2% parasitemia with *Babesia duncani*. One day before the start, media was changed, and parasitemia assessed by Giemsa smear as previously described.

Experimental Compounds

The following compounds were purchased from the respective supplier: Atovaquone (Thermo Scientific, catalogue #468762500); Azithromycin (Tokyo Chemical Industry (TCI), catalogue #A2076); Clindamycin (TCI, catalogue #C2257); Orlistat (Cayman Chemical Company, catalogue #10005426); Puromycin (Sigma, catalogue #P5057); Quinine (TCI, catalogue # Q0028); WR99210 (Sigma, catalogue #SML2976). Compounds in Series A, B, C, D, E, and F were obtained through Huston laboratory collaborations.

Diluting Parasitemia Experiments

Fresh A+ blood was washed three times with Puck's Saline Glucose + (PSG+) and a stock 10% hematocrit solution in cDMEM was created. In a 96-well plate, pellet from a vigorously growing (≥20% parasitemia) culture was used to create a 10% hematocrit, "100% parasitemia" starting well that was serially diluted five times with the stock 10% hematocrit solution. 25µL of

each serial dilution was transferred to a white, clearbottom 384-well plate (Falcon®, catalogue #353963) in triplicates (quadruplicates for 0% parasitemia), before 25µL of cDMEM was added to each well followed by 25µL of 0.6% Triton-X, 22.5µM Propidium Iodide (PI) in 1x Phophate Buffer Solution, no Ca^{2+} or Mg^{2+} (PBS). The 384-well plate was then shaken on fast for 30 seconds in a Powerwave XS (BioTek, Winooski, VT) before being incubated at 37ºC, 5% CO_2 for one hour then moved to room temperature in the dark. After 24-hours, the plate was read using a Synergy H1 (BioTek, Winooski, VT) with excitation = 535nm, emission = 615nm, sensitivity = 150, and readings taken from the bottom of the wells. Smears were made of the "100 parasitemia" starting well, stained as previously described in Chapter One and a minimum of 1,000 RBCs were read.

Z' Score Experiments and Calculation

To accurately calculate the Z' score, half of a 384-well plate was plated with 10% hematocrit, 5% parasitemia and treated with 50 µM WR99210 (0.5% dimethyl sulfoxide (DMSO)) (final 25 µM WR99210, 0.25% DMSO) in cDMEM while the other half was plated with 10% hematocrit, 5% parasitemia and treated with 0. 5% DMSO (final 0.25% DMSO) in cDMEM. The plate was incubated for 48-hours, after which a smear from each condition was made to assess parasitemia and the plate was read as previously described. Parasitemia was determined by counting a minimum of 1,000 RBCs in a condition blinded manner after smears were stained as previously described in Chapter One.

Z' scores for the assay were calculated using the described equation in Zhang et al., 1999[15] seen below.

$$Z' = \frac{3 \cdot \sigma_{Infected\ wells} + 3 \cdot \sigma_{Treated\ Infected\ wells}}{\left|\mu_{Infected\ wells} + \mu_{Treated\ Infected\ wells}\right|}$$

Compound Testing

Prior to the removal of the culture(s) from the modular incubator, fresh A+ blood was washed three times with PSG+ and compounds to be tested were prepared in a 96-well plate by serial dilution in cDMEM. Once prepared, the culture(s) were removed from modular incubator, collected, and washed once with 7mL of cDMEM. A 10% hematocrit, 5% parasitemia stock in cDMEM was made and 25µL aliquoted into a 384-well plate with constant agitation followed by 25µL of compound (quadruplicates). The positive growth control was given 25µL of 0.5% dimethyl sulfoxide (DMSO) in cDMEM (final 0.25% DMSO) and the negative growth control 25µL of 50 µM WR99210 (0.5% DMSO) (final 25 µM WR99210, 0.25% DMSO). The plate was incubated as previously described, smears of the positive growth control were made, and the plate was read as previously described. Smears were stained as previously described in Chapter One and a minimum of 1,000 RBCs were counted to determine parasitemia.

Data Handling and Analysis

The raw relative fluorescent units (RFU) output from Gen5 version 1.11 were imported into Microsoft Excel for data organization and analysis. GraphPad Prism version 10.0.2 (GraphPad Software, San Diego, CA) was used to generate dose-response curves (see below) and graphs.

To generate the simple linear regression, Prism default settings were used along with the option to consider each replicate Y value as an individual point.

To generate dose-response curves, the RFUs of a given experimental compound concentration were averaged (n = 4 wells) followed by subtraction of the negative growth controls (n = 28 wells) and expression of that as a percentage of difference between the positive growth control (n = 27 wells) and negative growth control. The percent inhibition values from at least two biological replicates were used to generate the presented EC_{50} values. EC_{50} values were calculated in GraphPad Prism as per the following equation, with the top constrained to 100: Y = Bottom +

(Top − Bottom)/(1 + $10^{LogEC50}$ − X × Hill slope), where Y is the percent inhibition, X is the drug concentration, and Hill slope is the largest absolute value of the slope of the curve.

Results

To find a reproducible method of linearly correlating parasitemia to relative fluorescent units (RFU) in a 384 well plate, the published *Plasmodium* SYBR Green was attempted as described in a 96-well plate[19,20] before miniaturization. This protocol was chosen over published *Babesia duncani* versions due to it being the original protocol and having an associated Z' score. Parasitemia and RFU did not correlate linearly at all when performed on infected red blood cells (iRBCs) that had been serially diluted with uninfected RBCs (uRBCs; Figure 1 Supplemental Three). This indicated that the protocol was potentially not suitable for miniaturization and use in a high throughput compound screen for *Babesia duncani*. To understand if the lack-luster results were due to a sensitivity issue with SYBR Green, several other fluorescent dyes were assessed. In a simple test of iRBCs vs uRBCs with the fluorescent dyes at the same concentrations, propidium iodide (PI) produced a significant difference by Welch's t-test and had the highest signal to background (Figure 2 Supplemental Three). SYBR Green did not produce a significant difference by Welch's t-test and gave the opposite of the expected result, with a higher RFU in the uninfected than in the infected conditions (Figure 2 Supplemental Three). Diluting parasitemia experiments were repeated to improve the modified protocol using PI until the final protocol herein was obtained.

To understand how well parasitemia and RFU correlated in the final protocol, a final set of diluting parasitemia experiments were performed. Figure 10 A shows that RFU correlates linearly with parasitemia. A simple linear regression was performed on the two independently repeated experiments and the r^2 was 0.95, indicating a very strong correlation. To determine the limit of

detection of the final protocol, the signal to noise (S/N) ratio was calculated using uRBCs as the blank value and values plotted. Figure 10 B shows the resultant graph. A simple linear regression was performed, and the resulting equation was used to calculate the limit of detection, which is where the S/N ratio is 1[15]. The limit of detection given was roughly 2% parasitemia.

To assess how the readout would work when in an assay, Z' scores were calculated. Z' scores indicate how good or bad an assay is as well as how good or bad the readout of the assay is by quantifying the overlap between control and treated sample populations[15]. As the intended purpose of the assay is to screen compounds for their anti-Babesial activity, we utilized a compound with known inhibitory effects, WR99210[8,17], on *Babesia duncani*'s growth as a negative growth control. Wells were inoculated at 5% parasitemia with 5% hematocrit and left to grow for 48-hours before smears were made, PI was added, and RFU read. The culture conditions and timeframe were chosen for the assay because parasitemia doubles roughly every 24-hours[16], meaning the endpoint parasitemia should be around 20% parasitemia, 20-30% parasitemia is the maximum parasitemia of a standard culture (own observation), 5% hematocrit is standard[16], and 48-hours allows two replication cycles, allowing us to catch any compounds that cause delayed death. Figure 11 shows the Z' scores for the two repeats of 0.819 and 0.822 respectively. According to Zhang et al., 1999[15], a Z' score between 1 and 0.5 indicates that the "assay is excellent". Additionally, the tightness of the two repeats Z' scores indicates that the assay is very reproducible.

Before testing compounds, it was important to understand if the drug solvent, dimethyl sulfoxide (DMSO), inhibits *Babesia duncani* and if so, at what concentration. This was important as DMSO is a standard solvent for compounds and if it is toxic to *Babesia duncani* at some concentration then that must be avoided as a confounding variable. Additionally, some compounds are not as soluble as others in DMSO meaning the compound is less concentrated at its starting point in the dilution series and more DMSO is present. DMSO appears to be inhibiting growth

around 1% final concentration (Figure 3 Supplemental Three), which is four times more than the final concentration (0.25%) typically present in the well. To better understand if the observed effect of DMSO on *Babesia duncani* growth was on growth or due to DMSO impacting the readout of the assay, RFU from cDMEM with DMSO that was serially diluted was compared to cDMEM with no DMSO after the addition of PI. The presence of DMSO did not appear to have an impact on RFU as the r^2 of the simple linear regression was 0.03 despite the apparent correlation by eye (Figure 4 Supplemental Three). This indicates that higher concentrations of DMSO, ≥1% final, are inhibitory to *Babesia duncani* growth and this observed inhibition is not due to DMSO's impact on the assay's readout.

The utility of the assay was put on display with dose-response testing of compound "mini"-series with known targets present in the Huston lab obtained through various collaborations. In addition, other compounds were tested for their anti-Babesial activity, including the current recommended treatment for *Babesia duncani* of atovaquone, azithromycin, clindamycin, and quinine[3]. Table 3 summarizes the compounds tested, their putative targets, and the Effective Concentration (EC_{50}) values obtained using this protocol. Atovaquone, azithromycin, clindamycin, and quinine had EC_{50} values of 0.517 μM, 12.4 μM, non-inhibitory (max concentration tested 5 mM), and 17.5 μM respectively. Several of the "mini"-series putative targets are theorized drug targets for *Babesia duncani*[16]. The most potent compound, F5 with an EC_{50} of 0.253 μM, was in series F that targets the cleavage and polyadenylation specificity factor (CPSF) complex. Phosphatidylinositol 4-Kinase (PI4K) inhibited by series E compounds showed less promise as the most potent compound was E1 with an EC_{50} of 11.0 μM. On the other hand, the lysine tRNA synthesis process targeted by series D, had a somewhat potent compound in D5 with an EC_{50} of 3.02 μM. Of the non-theorized targets, several potential new targets were identified for drug development. Series C, which inhibits the methionine tRNA synthesis process, one and only

compound C1 was potent with an EC_{50} value of 0.333 µM. Phosphodiesterases, which were inhibited by series B, had a wide range of EC_{50} values, potentially due to the different classes of phosphodiesterases present, with the most potent EC_{50} value 1.12 coming from B6. Series A targets an unknown protein, or family of proteins, but proved to be effective with A4 having the lowest EC_{50} value of 1.06 µM.

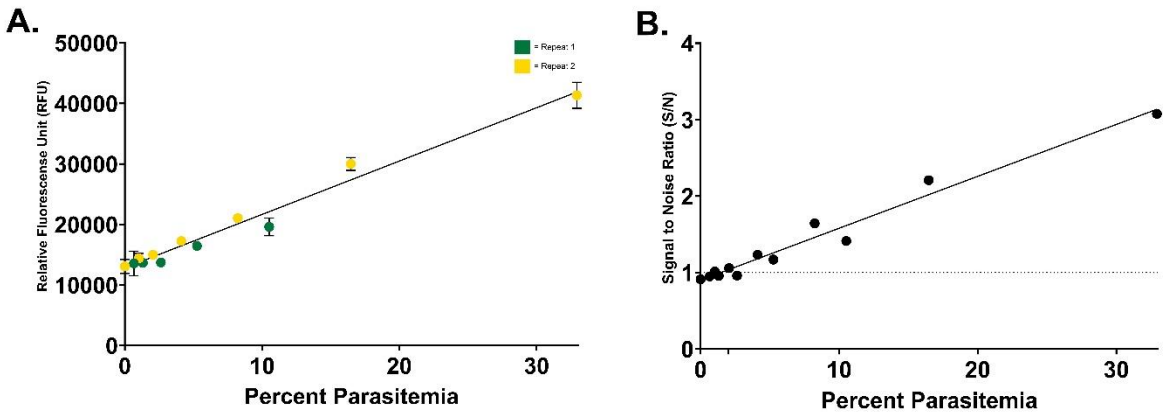

Figure 10 – Parasitemia and Relative Fluorescence Unit (RFU) Correlate Linearly with a Limit of Detection around 2% Parasitemia. A, Parasitemia and RFU correlate linearly, with an r^2 value of 0.95. B, 2% parasitemia, which is indicated at the tick-mark, is roughly the limit of detection (>1 S/N ratio[15]). The highest parasitemia of repeat 1 was excluded from analysis due to addition of too much staining solution. Parasitemia dilutions were prepared in a 96-well plate, transferred to a white clear bottom 384-well plate before cells were lysed with a 0.6% Triton-X, 22.5µM Propidium Iodide (PI) in 1x Phophate Buffer Solution, no Ca2+ or Mg2+ (PBS) staining solution and incubated. Fluorescence was 24 hours later from the bottom of each well, plotted in Prism against parasitemia, and a simple linear regression considering each replicate value as an individual point was performed. Signal to Noise (S/N) was calculated using 0% parasitemia as the background and limit of detection (1 S/N)[15] was calculated using the simple linear regression.

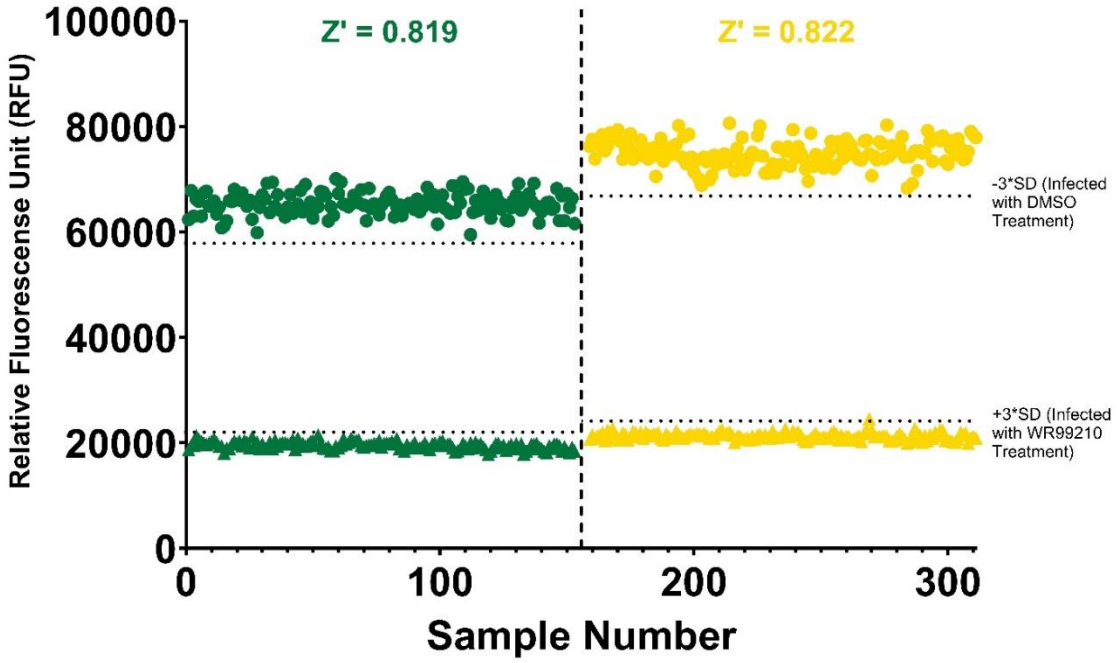

Figure 11 – Z' Scores Generated indicate that the Assay is Robust and Reproducible for High-Throughput Compound Screening. 5% parasitemia, 10% hematocrit were plated and treated with either 50 µM WR99210 (0.25% dimethyl sulfoxide (DMSO)) (triangles, n = 154) or 0.25% DMSO (circles, n = 154) for 48-hours. Plates were processed and Z' scores generated as previously described. Dashed lines represent the upper and low separation bands[15] respectively, the upper separation band is equal to $\mu_{\text{Infected DMSO Treated wells}} - 3\sigma_{\text{Infected DMSO Treated wells}}$ and the lower separation band is equal to $\mu_{\text{Infected WR99210 Treated wells}} + 3\sigma_{\text{Infected WR99210 Treated wells}}$. The vertical dotted line separate data from each of the two experiments which were performed on separate days.

Table 3 – Compounds Tested, Their Putative Targets, and Effective Concentration (EC_{50}) Values. A handful of compounds were tested using the developed assay to assess its ability to work in a high-through compound screening environment. Compounds screened included current *Babesia duncani* treatments of atovaquone, azithromycin, clindamycin, and quinine[3], compounds with known *Babesia duncani* efficacy, like WR99210[8], from the literature, and compound "mini"-series obtained through Huston lab collaborations. EC_{50} values were generated using the compound screening method as previously described in two separate repeats.

Compound	Putative Target	EC_{50} in µM (95% CI)
Atovaquone	Cytochrome b	0.517 (0.475 to 0.562)
Azithromycin	Apicoplast ribosomes	12.4 (10.3 to 14.9)
Clindamycin	Apicoplast ribosomes	Not effective (highest concentration tested 5mM)
Orlistat	Lipases	4.32 (3.40 to 5.50)
Paromomycin	Ribosomes	Not effective (highest concentration tested 5mM)
Quinine	Unknown	17.5 (15.8 to 19.3)
WR99210	Dihydrofolate reductase	8.32^{-3} (6.88^{-3} to ???)
A1	Unknown	10.7 (10.0 to 11.5)
A2	Unknown	1.53 (1.40 to 1.66)
A3	Unknown	2.90 (2.61 to 3.21)

A4	Unknown	1.10 (0.97 to 1.16)
A5	Unknown	192 (78.6 to 467)
B1	Phosphodiesterase (PDE)	21.5 (19.4 to 23.8)
B2	PDE	4.47^{9} (8.35^{-6} to 2.40^{24})
B3	PDE	10.6 (9.65 to 11.6)
B4	PDE	17.4 (15.0 to 20.1)
B5	PDE	5.65 (4.96 to 6.43)
B6	PDE	1.12 (0.898 to 1.40)
C1	Methionine tRNA Synthesis	0.333 (0.293 to 0.379)
D1	Lysine tRNA Synthesis	150 (11.7 to 1915)
D2	Lysine tRNA Synthesis	61.6 (56.5 to 67.1)
D3	Lysine tRNA Synthesis	21.8 (19.8 to 23.9)
D4	Lysine tRNA Synthesis	13.9 (12.5 to 15.5)
D5	Lysine tRNA Synthesis	3.02 (2.56 to 3.56)
D6	Lysine tRNA Synthesis	49.4 (46.3 to 52.8)
E1	Phosphatidylinositol 4-Kinase (PI4K)	11.0 (9.27 to 12.9)
E2	PI4K	36.5 (32.9 to 40.6)
E3	PI4K	926 (7.88 to 1.09^{5})
E4	PI4K	4.61^{4} (1.72^{-4} to 1.24^{13})
F1	Cleavage and polyadenylation specificity factor (CPSF) complex	1.47 (1.03 to 2.10)
F2	Cleavage and polyadenylation specificity factor (CPSF) complex	55.3 (47.6 to 64.2)
F3	Cleavage and polyadenylation specificity factor (CPSF) complex	2.76 (2.27 to 3.37)
F4	Cleavage and polyadenylation specificity factor (CPSF) complex	0.361 (0.290 to 0.450)
F5	Cleavage and polyadenylation specificity factor (CPSF) complex	0.253 (0.195 to 0.327)

Discussion

The only compound screening and testing method available for *Babesia duncani* is slow and tedious, dramatically hindering the drug pipeline which is needed for this emerging and geographically expanding pathogen[1,2,3,4,5]. Herein is a fast, robust, and reproducible, 384-well format assay for compounds that inhibit *Babesia duncani* growth. The diluting parasitemia experiments showed relative fluorescent units (RFU) correlated linearly with parasitemia, with a

reliable limit of detection of uninfected (0% parasitemia) versus infected at around 2% parasitemia. The Z' scores across two experiments were 0.819 and 0.822 respectively, indicating that the "assay is excellent"[15] and reproducible across repeats. This Z' score indicates that the assay is suitable for high-throughput screening of compound libraries to identify starting points for drug development or for use as chemical biologic probes. As initial proof-of-concept, we tested known *Babesia duncani* inhibitors in dose-response curves, and a small collection of compounds in development for the related parasite *Cryptosporidium*. Of the known *Babesia duncani* inhibitors, we got similar Effective Concentration (EC_{50}) values of reported compounds for *Babesia duncani*[16], further validating the assay. Additionally, amongst the target-based anti-*Cryptosporidium* compounds tested, several potent *Babesia duncani* inhibitors were identified that may indicate future drug development avenues.

There is a clear need for the standardized, robust, and methodically easy HTS format described herein for the *Babesia duncani* research field. Currently the research field utilizes a 96-well format SYBR Green I protocol[6,7,8,9,16,17,18] that is not high throughput/time efficient, standardized, or cost effective. The use of a 96-well plate for compound screening is simply not suitable for HTS, especially with such a large number of dilutions of a compound tested in three experiments[6,7,8,9,16,17,18]. While testing a large number of dilutions may be beneficial if the compound is especially potent, in the assay presented herein, dilutions are adjusted after the first replicate if the compound is found to be potent. Additionally, the fact a 96-well plate is not suitable for HTS is further put on display by the logistics of a library screen. Typically, in a library screen the compounds are tested at 5µM and 1µM in quadruplicate wells with those inhibiting at both concentrations moving forward to dose response curve. If done solely in 96-well plates, and assuming no controls, roughly five (5.1) 96-well plates would be needed to equal one 384-well in terms of the compounds that can be screened. The SYBR Green assay used by *Babesia duncani*

researchers is not standardized, not across the field nor even in specific laboratories[6,7,8,9,16,17,18]. The original SYBR green assay was described in *Plasmodium falciparium*[19,20] and is not faithfully reproduced in any of the *Babesia duncani* literature[6,7,8,9,16,17,18]. This is problematic as the deviation from the *Plasmodium* protocol necessitates the generation of a Z' score, especially with the changes to key elements of the protocols such as: omission of the freeze-thaw step[6,7,8,17,18]; change in starting parasitemia[6,7,8,17,18]; change in the hematocrit[6,7,8,16,17,18]; change in compound treatment time[6,7,8,16,17,18]; and incubation time after addition of SYBR Green lysis solution[6,7,8]. Additionally, even within laboratories the protocol is not standardized and has changed noticeably between publications[6,7,8,16,17]. A final consideration for this HTS assay over the SYBR Green among all the previously mentioned points is the cost effectiveness of it. The need to utilize more 96-well plates to equal a 384-well plate makes the 384-well plate more cost effective per plate. Additionally, half as much as volume (50µL) is used in the 384-well plate as is used in a 96-well plate (100µL). This is particularly helpful as cDMEM is not a cheap media to complete due to the additives it requires[16]. The amount of compound being tested is greatly reduced as well by the miniaturization to a 384-well plate, which is helpful for testing difficult or expensive to synthesize compounds. Lastly in the consideration of cost effectiveness is the DNA dye used, SYBR Green I vs propidium iodide. SYBR Green I costs $0.67 per µL for a 1mL tube[21] compared to the propidium iodide used for this work that costs $0.0096 per µL for a 10mL bottle[22].

We have tested compounds with a range of likely drug targets against *Babesia duncani* and found several hits. Many of these have been known to *Babesia duncani,* thus further validating our assay, theorized as targets, or are novel. Atovaquone, azithromycin, clindamycin, quinine and WR99210 are all known drugs to *Babesia duncani*[8,16,17]. Notably a few of our generated EC_{50} values differed from those in the literatures: clindamycin (inactive vs 20 µM[16]); quinine (17.5 µM vs 12 µM[16]); and WR99210 (8 nM vs 0.5 nM[17]). These differences may be due to the assay, supplier of

the compound, or analysis of the data as noticeably for clindamycin and quinine, the researchers artificially treated the highest concentration of compounds (100μM) as 100% inhibition of parasite growth[16]. There is no proof given in the paper[16] that this is the case and would skew any dose response curves, as well as EC_{50} values generated for the compounds.

Our compound testing confirmed that several previously theorized[16] *Babesia duncani* drug targets may be viable therapeutic targets while also suggesting additional potential targets. Compound series D, which targets lysine tRNA synthesis, most potent compound was D5 with an EC_{50} of 3.02 μM. Like *Plasmodium* and other apicomplexans, *Babesia* spp. lack de novo amino acid synthesis pathways and scavenge for amino acids in their hosts[23]. This makes the tRNA synthesis process a perfect drug target as it is essential for parasite growth and although the process is conserved in the host, the enzymes will have evolved to allow parasite specificity. Compound series E, which targets phosphatidylinositol 4-kinase (PI4K), had limited efficacy with the most potent compound being E1 with an EC_{50} of 11.0 μM. PI4Ks are lipid kinases that phosphorylate phosphatidylinositol to generate phosphatidylinositol 4-phosphate (PI4P). In *Plasmodium*, PI4Ks are believed to drive cytokinesis events as treatment with PI4K inhibitors prevents formation of individual schizonts in RBCs[24]. Despite the lackluster results of the compounds in the series presented herein, there has been promising data published in the literature for PI4K targeting compounds in the treatment of human babesiosis for *Babesia microti* in mouse models[25]. Compound series F, cleavage and polyadenylation specificity factor (CPSF) complex inhibitors, had two potent inhibitors, F4 and F5 with EC_{50} values of 0.361 μM and 0.253 μM respectively. CPSFs as part of the whole complex are involved in the recognition and cleavage of the poly-adenylation signal from the 3' end of pre-mRNA in its maturation to mRNA[26]. This makes CPSFs, and the whole complex, an excellent drug target as transcription is an essential process for life. More research is required however into their mechanism of action as currently it is unclear exactly

how these compounds are working and if the compounds are working on the complex or on individual CPSFs[27].

The novel potential drug targets hold the most potential intrigue as these are unexplored and provide ample area for drug development. Orlistat is a reversible lipase inhibitor that covalently binds to the conserved serine residue in the active site[28]. Herein is the first report of Orlistat inhibiting a *Babesia* spp. growth, with our EC_{50} value for *Babesia duncani* being 4.32 μM. It is unclear at this time if Orlistat is acting on an RBC lipase which is preventing growth of *Babesia duncani* or if Orlistat is acting on *Babesia duncani* lipases. It seems more likely that Orlistat is working on *Babesia duncani* lipases as RBCs primarily run on glycolysis, although there has been some recent data to suggest RBC utilize fatty acids to various capacities[29]. There are significant drops in low-density lipids (LDLs) and high-density lipids (HDLs) in individuals with babesiosis[30], indicating either metabolism of LDLs and HDLs by the parasite or a change in their levels due to disease. Taken all together, along with the report that Orlistat has nanomolar potency in Plasmodium while acting on a lipase[31] as well patients infected with *Plasmodium falciparum* experiencing the same drop in LDLs and HDLs[32], indicate that Orlistat is most likely acting on *Babesia duncani* lipases and these lipases are novel drug targets. Compound series A has an unknown target but had two potent compounds in A4, EC_{50} of 1.06 μM, and A2, EC_{50} of 1.53 μM. While serving as leads for development, these compounds could be used to generate resistance in *Babesia duncani* to identify the target. Once resistance is generated and the target identified, CRISPR/Cas9 genome editing could be used to verify the mutation causes resistance. Unfortunately, despite being the main focus of this thesis, CRISPR/Cas9 is not functional to perform this type of work at this time. In lieu, enzymatic assays with a wild type and resistance carrying enzyme could be performed or an episomal transfection could be attempted to see if resistance to either compound is recapitulated. Compound series B, which target

phosphodiesterases (PDEs), was a mixed bag with B6 as the most potent compound with an EC_{50} of 1.12 μM. Potentially owing to this fact is that PDEs cleave phosphodiester bonds, with the specific phosphodiester bond cleaved typically referring to the one present in cAMP and cGMP but there being other phosphodiester bonds cleaved. Not much is known about the role of PDEs in *Babesia*. In *Babesia divergens,* treatment with the cGMP-PDE inhibitor BIPPO induced motility, treatment with cGMP analog 8-Br-cGMP induced egress, and a CRISPR/Cas9 knockdown system of protein kinase A revealed cAMP may be required for invasion[33]. This recapitulates what is seen in *Plasmodium falciparum*, where the compound BIPPO was discovered[33]. Compound series C, which targets methionine tRNA synthesis, only had one compound which turned out to be potent with an EC_{50} value of 0.333 μM. As mentioned previously *Babesia* spp. lack de novo amino acid synthesis pathways therefore scavenge for amino acids from their hosts[23]. This makes any tRNA synthesis process an intriguing drug target as it is essential for life and the involved enzymes will more than likely differ from the host's own tRNA synthesis enzymes.

Looking outwards to other holes in the *Babesia duncani* drug pipeline, there are other assays that need to be developed such as; invasion and egress assay. Both are important events in the lifecycle of *Babesia duncani* and would help narrow down the window of the compound is active. More importantly, it might help to remove some compounds from further development as if it is a compound is found to block egress, it will more than likely be an unviable drug. This is because most individuals who present with babesiosis are asplenic[1,2,3] and without a spleen, these infected red blood cells (iRBC) are less likely to be removed from the blood circulation. Additionally, while egress may be blocked, the parasites will most likely be metabolically active which dramatically increases the chances of drug resistance occurring. Both assays would utilize flow cytometry or fluorescent microscopy as the primary readout. For the invasion assay, isolated *Babesia duncani* merozoites would be incubated with the drug for a set time before being added to

a solution with RBCs for an hour to allow invasion. The cells would be washed, stained, and percent invasion inhibition determined for the compounds tested with the positive invasion inhibitor heparin[33]. This assay could be adapted to test if the compound is working on the cell or parasite side by treating the RBC with the compound then allowing the *Babesia duncani* merozoite to invade[34,35,36]. If no effect is seen when the compound is incubated with the RBC then the compound is most likely working on the *Babesia duncani* merozoite but if an effect is seen, the compound is most likely disrupting the cell's pathway *Babesia duncani* utilizes to invade[34,35,36]. Additionally, the assay could be used to screen antibodies, either patient derived from potential vaccines or from a commercial source, for their effectiveness. For the egress assay, high parasitemia (>10-15%) iRBCs would be incubated with the compound alongside heparin[33], to prevent reinvasion, for a set time. The cells would be washed, stained, and percent egress inhibition determined for the compounds tested with the positive egress inducer 8-Br-cGMP[37] and the negative egress inducer E-64[38].

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Chapter Five – Summary

In the thesis work presented herein: *histone 2B* (H2B) promoter was identified as the strongest promoter tested, making it potentially suitable for constitutive expression of transfected genes although an *in vitro* experiment needs to performed to confirm its usefulness; it was discovered *Babesia duncani* can spontaneously pick up DNA present in red blood cells which was exploited to optimize a transfection protocol (Chapter Two); the optimized transfection protocol was used for the first reported attempt of a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 transfection in *Babesia duncani* with promising results (Chapter Three); and a 384-well high throughput compound screening format was developed with which a handful of compounds (Chapter Four), as well as sugars and lectins (Supplemental Two), were tested for their anti-babesial activity, identifying several avenues for potential therapeutic development and novel selectable markers.

As it stands the CRISPR/Cas9 system is non-functional and requires work. Potentially this is as simple as working out an optimal protocol to select transgenic parasites or improving expression of *Streptococcus pyogenes* Cas9 (SpCas9) and repair cassette encoded on TPXgRNA_GB3_CC9 and TPX_FsdRC_GB3 respectively under *H2B* or *eF-1α* instead of *actin* as it does not have a strong promoter (Chapter One). Additionally, increasing the number of parasites used in the transfection would be beneficial as this increased the number of transfected *Babesia duncani* (Chapter Two). Once there is a working CRISPR/Cas9 system, the system could be used to knockout genes to explore gene functions and host-pathogen interaction; create reporter parasites for *in vivo* and *in vitro* assays or needs; drug target identification validations; and creation of attenuated strains for vaccine or immunotherapy purposes.

As *Babesia* spp. lack non-homologous end joining (NHEJ)[1], one of the most fruitful avenues would be to create a transgenic *Babesia duncani* that is capable of NHEJ. This would allow

easy knockout of genes as a repair cassette would not need to be included in the transfection, meaning the transgenic *Babesia duncani* could be used in phenotypic library screens of gene functions, drug target identification validation by mutagenesis, or creation of attenuated strains by mutagenesis of key virulence proteins. Another fruitful avenue would be the adaptation of the CRISPR/Cas9 system to the other Cas systems, such as Cas13 or Cas12. Cas13 targets ssRNA[2] and could be used as a way to conditionally knockdown genes if a *Babesia duncani* was made to be constitutively expressing it and fed gRNA as needed. Cas12 targets DNA[2] and has been described as a fast, sensitive and specific diagnostic tool[3], which *Babesia duncani* could use. One final fruitful avenue would be the creation of generic reporter strains for *in vitro* and *in vivo* drug assays to aid the *Babesia duncani* drug development pipeline. The most immediate reporter strain created should be a fluorescent expressing *Babesia duncani* reporter strain. This was the goal of Chapter Three as it would allow omission of propidium iodide or any fluorescent dyes in the 384-well high throughput compound screening format described in Chapter Four. Additionally, this reporter strain may facilitate the miniaturization to a 1536-well plate, greatly increasing the number of compounds that can be screened among many other advantages discussed in Chapter Four. Another immediate reporter that should be created to aid the *Babesia duncani* drug development pipeline is a luciferase expressing parasite that can be for *in vivo* drug efficacy tests. This reporter parasite would replace the need for tedious, human-error prone, and daily Giemsa stains to assess parasitemia during drug treatment while being more sensitive. Other reporters that may be beneficial due to their ability to accelerate drug development but are currently unattainable due to the poorly annotated genome would be fluorescent reporters that are under the control of specific stress response genes (i.e. oxidative stress, DNA damage, unfolded protein accumulation, metabolic stress, etc.). These reporter strains have been theorized, realized, and used in bacteria to aid in identifying the mechanism of action of current and novel antibiotics[4,5]. If realized for *Babesia duncani*, these types

of reporter parasites would undoubtedly be of great utility to drug development and to the research field.

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Supplemental One – Primers and Plasmids Used

Table 1. List of Primers, Respective Sequence, and Purpose used in this Thesis.

Name	Sequence (5' to 3')	Purpose
eF-1-Alpha Forward	GCTGGTACGACAAGCATAGC	Promoter strength
eF-1-Alpha Reverse	GCTCCTTCAAATACGCTTGGG	Promoter strength
Aldolase Forward	TGATGCCTTGTGCATGCAAC	Promoter strength
Aldolase Reverse	ACTGGGACCATCAAGAAGCG	Promoter strength
H2A Forward	AGGCGGTAAAGGAAAGACGG	Promoter strength
H2A Reverse	GTTGAACCAACACGCCCATC	Promoter strength
HSP70 (Chrom5) Forward	CTTGGTTTGGAAACCGCTGG	Promoter strength
HSP70 (Chrom5) Reverse	GTGCAATGCCAGAAAGGTGG	Promoter strength
Alpha Tubulin Forward	TCAGCCTGATGGGCATATGC	Promoter strength
Alpha Tubulin Reverse	TCTCACCTCGTCAACAACCG	Promoter strength
Pyruvate Kinase Forward	AGGCGAGTCCCAAGGATTTG	Promoter strength
Pyruvate Kinase Reverse	GGACGCCTCTTGATAGCCTC	Promoter strength
HSP70 (Chrom4) Forward	CGCGATGCACTTGATGAAGG	Promoter strength
HSP70 (Chrom4) Reverse	GCTGTATCCAGAGTCGTCCG	Promoter strength
Enolase Forward	TCCAATCCGGTTACACCAGC	Promoter strength
Enolase Reverse	TGGGAATCCAACCGTTGAGG	Promoter strength
H2B Forward	GCGTGCTTGGATAGTTCACC	Promoter strength + transfection readout + PCR
H2B Reverse	TCAAGCAGGTTCACCCTGAC	Promoter strength + transfection readout + PCR
Casein Kinase 1 Forward	GTCTACCGTGGCAAGGTCTC	Promoter strength
Casein Kinase 1 Reverse	GGTCCTTGAGTATCCTGCGG	Promoter strength
HSP90 Forward	GATGACGAGGCCGAAGAGAG	Promoter strength
HSP90 Reverse	GGTGAACGCATCCAAATGGG	Promoter strength
Actin Forward	TTGCGGTGAACAATGTTGGG	Promoter strength
Actin Reverse	TGGTCAGCGTATGACCAAGG	Promoter strength
LDH Forward	TGCTCAAGTCCACTGGATTCC	Promoter strength
LDH Reverse	GAGTGGGAGTCCGTTGACAG	Promoter strength
Myosin A Forward	TATCCGCCATGGCTCCATTG	Promoter strength
Myosin A Reverse	TTCCAGGCACATCTAGGCAC	Promoter strength
G6PI Forward	AAGGGAACAGGCCATCCATG	Promoter strength
G6PI Reverse	TACCTTGCCCAGTTCAACCC	Promoter strength
GB4_NLuc Forward	TGAGCAAGGAGGTGTGTCAAGT	Transfection readout
GB4_NLuc Reverse	GTCGTCCACAGGGTAAACCACT	Transfection readout
TPX_FsdRC_GB3_Lin_For	CAGGAAACAGCTATGACCAT	Linearize TPX_FsdRC_GB3
TPX_FsdRC_GB3_Lin_Rev	TGTAAAACGACGGCCAGTGA	Linearize TPX_FsdRC_GB3
TPXgRNA_GB3_CC9_PCR_For	ACTTACCATGACTTGTTGAAGA	PCR

TPXgRNA_GB3_CC9_PCR_Rev	CGACTCATTGACATTTAAGGAG	PCR

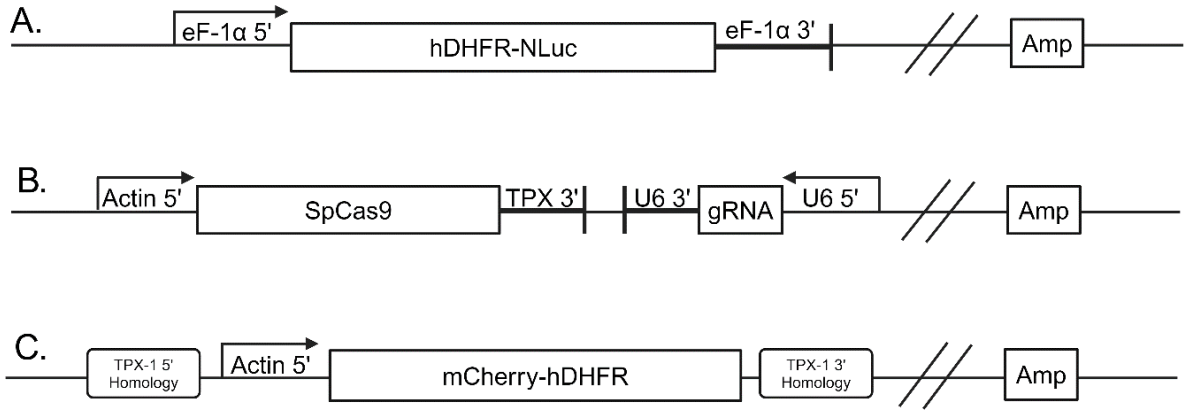

Figure 1. Schematics of Plasmids used in this Thesis. A, GB4_NLuc encodes a *human dihydrate folate reductase* (hDHFR) linked to *nanoluciferase* (NLuc) by a $(GGGS)_2$ linker driven by *elongation factor 1 alpha*'s promoter (eF-1α 5'). The *hDHFR, NLuc*, and $(GGGS)_2$ are *Babesia duncani* codon optimized. B, TPXgRNA_GB3_CC9 encodes *S. pyogenes* Cas9 (SpCas9) driven by *actin*'s promoter (Actin 5') convergent to the guide RNA (gRNA) driven by *U6 spliceosomal RNA*'s promoter (U6 5'). SpCas9 is *Babesia bovis* codon optimized and *actin*'s promoter was chosen to drive SpCas's expression because at the time codon usage for *B. duncani* was not available, the RT-qPCR data from Chapter One had not been generated yet, and *actin* is highly expressed in related Apicomplexa. *Babesia duncan*i's *U6* was identified by performing a NCBI BLAST of *Plasmodium falciparium*'s *U6 spliceosomal RNA* and *Toxoplasma gondii U6 snRNA*[1] against *B. duncani*'s genome. C, TPX_FsdRC_GB3 encodes a *mCherry* linked to a *human dihydrate folate reductase* (hDHFR) by a (GGG) linker driven by *actin*'s promoter (Actin 5'). 70 base pairs of *thioredoxin-1* (TPX-1) homology are present to help direct homologous recombination once Cas9 makes a cut. *mCherry* and *hDHFR* are *B. bovis* codon optimized and *actin*'s promoter was chosen to drive the fusion's expression because at the time codon usage for *B. duncani* was not available, the RT-qPCR data generated herein had not been generated yet, and *actin* is highly expressed in related Apicomplexa.

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Supplemental Two - Sugar Precipitation and Lectin Blocking to Identify *Babesia duncani* Sugar Binding Motif

Methods

Culture Maintenance and Culture Preparation for Testing

Culture maintenance was performed as previously described in Chapter One and Chapter Four.

Sugar Precipitation Assay

Sugar precipitation assays were performed using the same protocol as described for compound testing in Chapter Four. Sugars tested included: Arabinose; Fucose; D-Galactose; Glucose; Lactose; Maltose; Mannitol; Mannose; N-acetyl-D-galactosamine; N-acetyl-D-glucosamine; Sucrose; Trehalose; and D-(+)-xylose.

Red Blood Cell (RBC) Lysis Assay

RBC lysis assay was performed using a modified protocol of the compound testing as described in Chapter Four. Briefly, fresh A+ blood was washed three times with PSG+ and sugars to be screened were prepared in a 96-well plate by serial dilution in cDMEM. Once prepared, a 10% hematocrit, 0% parasitemia stock in cDMEM was made and 25µL aliquoted into a 384-well plate with constant agitation followed by 25µL of sugars. The positive lysis control was given 25µL of 10% Triton-X100 in cDMEM and the negative lysis control 25µL of cDMEM. The plate was incubated as previously described, and the plate was read by taking absorbance at 630nm using a Synergy H1 (BioTek, Winooski, VT).

Sugars tested included: Fucose; D-Galactose; Glucose; N-acetyl-D-galactosamine; N-acetyl-D-glucosamine; Sucrose; and Trehalose.

Lectin Blocking Assay

Lectin Blocking assays were performed using a modified protocol as described for compound testing as described in Chapter Four without a serial dilution. Each lectin (n = 28) was diluted to 1:250 in cDMEM and treated as a "compound". The experiment was performed only once. Lectins (Vector Laboratories, catalogue #BK-1000) tested included: Concanavalin A (ConA); Dolichos Biflorus Agglutinin (DBA); Peanut Agglutinin (PNA); Ricinus Communis Agglutinin (RCA); Sambucus Nigra Lectin (SNA); Soybean Aggulutin (SBA); Ulex Europaeus Agglutinin (UEA); Vicia Villosa Lectin (VVL); and Wheat Germ Agglutinin (WGA).

Data Handling and Analysis

As previously described for compound screening, % inhibition, and EC_{50} curve generation. % lysis and LC_{50} curve generation were the same as % inhibition and EC_{50} curve generation, respectively. GraphPad Prism version 10.0.2 (GraphPad Software, San Diego, CA) was used for data analysis.

Results

Table 1 shows the Effective Concentration (EC_{50}) and Lytic Concentration (LC_{50}) values of the sugars tested. The three sugars with the lowest EC_{50} were mannose, N-acetyl-D-glucosamine, and sucrose at 30.7 mM, 39.8 mM, and 40.7 mM respectively. To better understand if the observed EC_{50} values were due to host cell lysis or actual inhibition of parasite growth, LC_{50} were generated. LC_{50} values were generated by using the same experimental protocol setup as the compound screening except with uninfected cells and a positive lysis control (Triton X-100). N-acetyl-D-glucosamine and sucrose had a high LC_{50} at 8.69[82] mM and 5.31[38] mM respectively, indicating that the inhibition was due to inhibition of parasite growth, not due to host cell lysis. A LC_{50} value for

mannose was not generated therefore it is impossible to say if the observed EC_{50} value for mannose is due to host cell lysis or actual inhibition of parasite growth.

To probe the question from the opposite side, lectins were employed as they bind specific sugars with relatively high specificity. Of the lectins tested, table 2, at a final concentration of 1:500, only Ulex Europaeus Agglutinin (UEA) inhibited growth with the percent inhibition being 2.55%. UEA preferentially binds α-fucose[1], indicating it may be important. Unexpectedly, Ricinus Communis Agglutinin (RCA) and Wheat Germ Agglutinin (WGA) appeared to stimulate growth as their percent inhibition was -22.5% and -12.2% respectively. RCA binds to galactose and N-acetyl-D-galactosamine[1] while WGA binds to N-acetyl-D-glucosamine and sialic acid[1], indicating exclusion of these may be beneficial for some unknown reason.

Table 1. Effective Concentration (EC_{50}) and Lytic Concentration (LC_{50}) of Sugars tested. Mannose, N-acetyl-D-glucosamine, and sucrose had the lowest EC_{50} values of the sugars tested at 30.7mM, 39.75mM, and 40.72mM respectively. LC_{50} values of N-acetyl-D-glucosamine and sucrose were generated and indicate the concentration of sugars used are not lysing the RBCs to cause the observed EC_{50} values. The LC_{50} mannose was not generated. EC_{50} values were generated using the same protocol for compound screening. LC_{50} values were generated using a modified compound screening utilizing uninfected RBCs incubated with the various sugar concentrations, a positive lysis control of 5% Triton X-100, a negative lysis control of media, and the readout being absorbance at 630nm at 48-hours.

Sugar	EC_{50} in mM (95% CI)	LC_{50} in mM (95% CI)
Arabinose	128 (119 to 137)	Not Tested (NT)
Fucose	216 (187.9 to 248.3)	773 (378 to 1582)
D-Galactose	164 (147 to 181)	7272 (1024 to 5.17[5])
Glucose	84.0 (79.6 to 88.6)	1057 (366 to 3053)
Lactose	59.2 (54.4 to 64.4)	NT
Maltose	44.8 (41.20 to 48.61)	NT
Mannitol	74.6 (68.6 to 81.0)	NT
Mannose	30.7 (28.4 to 33.1)	NT
N-Acetyl-D-Galactosamine	62.4 (52.1 to 74.8)	infinity (Very wide)
N-Acetyl-D-Glucosamine	39.8 (32.7 to 48.4)	8.69[82] (Very wide)
Sucrose	40.7 (36.3 to 45.6)	5.31[38] (Very wide)
Trehalose	52.1 (46.2 to 58.8)	4.88[83] (Very wide)
D-(+)-Xylose	148 (119 to 184)	NT

Table 2. Percent Inhibition of Babesia duncani Growth of Lectins Tested. Ulex Europaeus Agglutinin (UEA) was the only lectin tested that inhibited Babesia duncani growth. Unexpectedly two lectins tested, Ricinus Communis Agglutinin (RCA) and Wheat Germ Agglutinin (WGA) produced strong negative percent inhibition. This may indicate they are promoting Babesia duncani growth by some unknown mechanism. Experiment was only performed once and

was performed using a modified compound screening protocol where the lectins were diluted to 1:250, wells (n = 28 per lectin) were treated for 48-hours, and plates read as previously described.

Lectin	% Inhibition (95% CI)
Concanavalin A (ConA)	-2.90 (-4.60 to -1.20)
Dolichos Biflorus Agglutinin (DBA)	-1.85 (-3.41 to -0.293)
Peanut Agglutinin (PNA)	-0.842 (-2.42 to 0.738)
Ricinus Communis Agglutinin (RCA)	-22.5 (-24.8 to -20.2)
Sambucus Nigra Lectin (SNA)	-2.90 (-5.71 to -0.085)
Soybean Aggulutin (SBA)	-2.63 (-4.07 to -1.19)
Ulex Europaeus Agglutinin (UEA)	2.55 (1.28 to 3.82)
Vicia Villosa Lectin (VVL)	-1.81 (-3.37 to -0.243)
Wheat Germ Agglutinin (WGA)	-12.2 (-15.8 to -8.66)

Discussion

It is currently unknown what proteins *Babesia duncani*, and more broadly *Babesia* spp., are recognizing on the surfaces of red blood cells (RBC) before invasion and conversely what *Babesia* proteins are responsible for this recognition. The work presented herein was undertaken to identify the sugar motif that *Babesia duncani* is recognizing on RBC cell surfaces for invasion in hopes that might lead to the discovery of those *Babesia duncani* proteins. Such a discovery would open avenues for targeted drug development, vaccine development, and therapeutic antibodies in the treatment of human babesiosis. The sugar precipitation assay yielded mannose, N-acetyl-D-glucosamine, and sucrose with the lowest Effective Concentration (EC_{50}) values of 30.7 mM, 39.8 mM, and 40.7 mM respectively. To exclude the possibility that the EC_{50} observed was due to host cell lysis induced by osmotic changes by the addition of the sugars, lytic concentration (LC_{50}) values were calculated for a handful of the sugars tested. N-acetyl-D-glucosamine and sucrose had LC_{50} values of 8.69[82] mM and 5.31[38] mM respectively, indicating the sugar concentrations tested were not lysing the RBC and the EC_{50} values obtained were due to inhibition of parasite growth.

Mannose's LC_{50} value was not calculated and therefore nothing can be said about if the EC_{50} value obtained was due to inhibition of parasite growth or lysis of the host cell. In addition to the sugars, a handful of lectins were screened for their ability to inhibit growth at a dilution of 1:500. Due note, this experiment was only performed once so the results presented herein should be taken as preliminary until a repeat can be performed. Ulex Europaeus Agglutinin (UEA) was the only lectin that inhibited growth, with inhibition of 2.55%. UEA binds preferentially to α-fucose[1], indicating fucose may be near the sugar motif but not the sugar motif due to the lectin's low inhibition and fucose high EC_{50} value. Interestingly, Ricinus Communis Agglutinin (RCA) and Wheat Germ Agglutinin (WGA) stimulated growth with percent inhibition of -22.5% and -12.2% respectively. RCA binds to galactose and N-acetyl-D-galactosamine[1] while WGA binds to N-acetyl-D-glucosamine and sialic acid[1]. Unfortunately, these results are more difficult to interpret as N-acetyl-D-galactosamine and N-acetyl-D-glucosamine had low EC_{50} values, meaning these results contradict each other as the LC_{50} values for both sugars were extremely high further indicating the inhibition is due to inhibition of parasite growth not lysis of host cells. It is possible the RCA and WGA create a "bridge" that *Babesia duncani* utilities to find the RBC more easily and its own sugar binding motif protein can out-compete the RCA/WGA once it is at the RBC cell surface. One way to confirm or deny this as a possibility would be to test multiple dilutions of the lectins. If *Babesia duncani* is using RCA/WGA as a "bridge" to find the RBC more easily, diluting out RCA/WGA further and further should reduce the stimulated growth seen as less "bridges" are present. Another possibility is that the RCA and WGA lectins are naturally fluorescent around 600nm, the emission used for this protocol. This possibility could be ruled out by testing the lectin by itself against nothing with and without propidium iodide to see if there is a spike in relative fluorescence unit (RFU) when the lectins are present.

The protocols used herein, specifically the sugar precipitation assay, were the first experiments that led to the discovery of *Entamoeba histolytica* Gal-lectin[2] and *Plasmodium*'s erythrocyte binding-like (EBL) protein family[3]. Both proteins have been the source of intense research as they hold the greatest potential as vaccines and therapeutics for their respective diseases, with preliminary data confirming their potential[4,5]. To isolate the *E. histolytica* Gal-lectin Petri et al., 1987[6] utilized affinity chromatography derivatized galactose and immune-blots with monoclonal antibodies that inhibited amoebic adherence. *Plasmodium* researchers utilized a similar strategy in Jungery et al., 1983[6] to isolate the EBLs with a size-exclusion column washed with sugars or RBC sialo-glycoproteins. A variation of these protocols was planned if an inhibitory sugar was identified to isolate and identify *Babesia duncani*'s protein(s) responsible for recognizing the RBC however such a sugar has not been identified.

Looking forward, the most fruitful line of research towards identifying and isolating *Babesia duncani*'s protein(s) responsible for recognizing the RBC would be to screen more sugars in the sugar precipitation assay. It should be noted however that the most potent sugar screened herein, mannose, had a similar EC_{50} to *Plasmodium*'s most potent sugar, N-acetyl-D-glucosamine, which was around 15 mM[3]. This potentially indicates the inhibiting sugar has been identified but as previously discussed, the LC_{50} for mannose was not generated therefore it is impossible to say if the EC_{50} value observed for mannose is due to inhibition of parasite growth or to host cell lysis. Additionally, host cell lysis may not be the best readout for overall cell health as *Babesia duncani* is an obligate intracellular parasite, host toxicity is most likely passed onwards to *Babesia duncani*, impacting its growth. Therefore, to truly understand if the observed percent inhibition is due to inhibition of growth or host toxicity, a better standardized cell health assay is needed. Such assays could utilize commercially available assays that measure L-lactate, an end product of glycolysis, or ATP levels in uninfected, sugar treated RBCs. If the lectin blocking approach is decided as a

more fruitful line of research, there are several considerations. Firstly, in this approach the lectins were treated as a compound, meaning they were added to the wells and left to bind to whatever is present in the media, on the RBC, or on the parasite. This may have the unintended consequence of binding solely to components in the media, thereby producing no observable effect, binding to the RBC, which would be the desired outcome, binding to the parasite as it egresses and invades a new RBC, thereby probably producing an erroneous percent inhibition, or some combination of the above. A more control methodology would be to treat a set of RBCs with a specific lectin, washed, then infected. This removes the possibility of the lectin binding solely to components in the media and binding to the parasite as it egresses and invades a new RBC. It does introduce the new possibility that no observable difference is seen despite this modified protocol because of the presence of non-lectin blocked RBCs from the infecting RBCs but this can be overcome by increasing the parasitemia to decrease the number of RBCs added to the lectin-blocked RBCs. The second consideration is the need for a sugar-lectin control for each lectin used. As each lectin binds a specific sugar, the proper control for each lectin is the incubation of the lectin with its known sugar during blocking. This dramatically increases the number of conditions and the concentration of sugar recommended for these controls is not provided by manufacturer/sellers[1]. A final consideration is the redundancy in the sugar binding motifs of available lectins[1]. This makes it difficult to use readily commercially available lectins for screening purposes like the one described herein. It may be more beneficial to use non-commercially available lectins, such as *E. histolytica* Gal-lectin or *Plasmodium*'s EBLs for the screening as these have well known targets and unique sugar binding motifs, especially in the case of *Plasmodium*'s EBLs[5].

Sources

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Supplemental Three – Chapter Four Supplemental Data

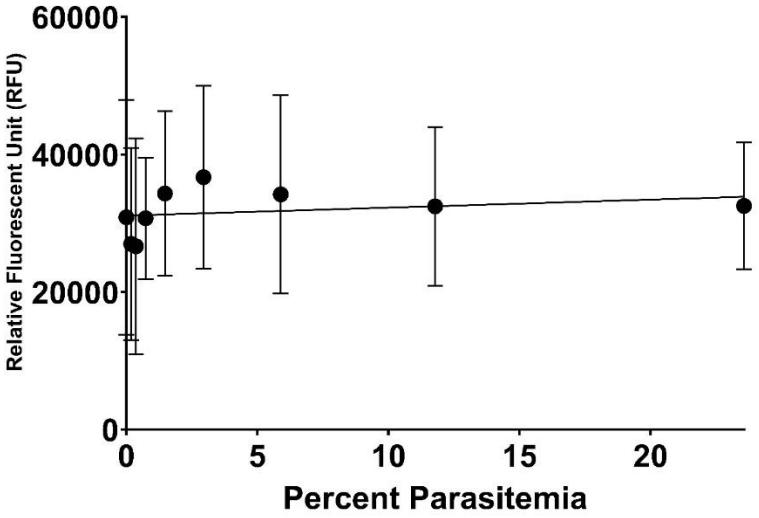

Figure 1. The Established SYBR Green Protocol does not Correlate Parasitemia with Relative Fluorescence Unit (RFU). A simple linear regression was performed and the r^2 was 0.005952, indicating there is no correlation between parasitemia and RFU. Parasitemia dilutions were prepared in 1.5mL tubes, transferred to a black clear bottom 96-well plate before cells were freeze-thawed, SYBR-Green lysis solution added, and the plate read as described in Jonhson, J., et al., 2007.

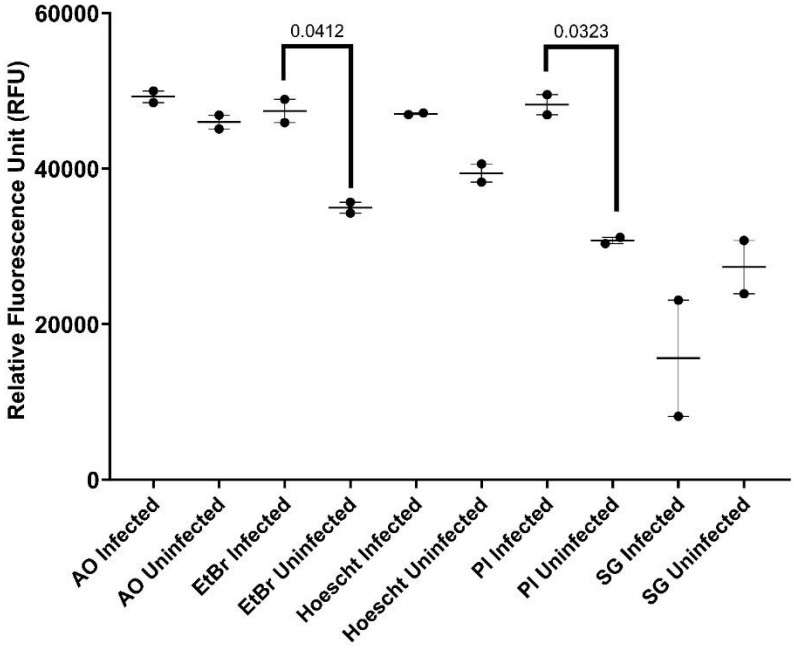

Figure 2. Propidium Iodide (PI) allows Discrimination of Infected vs Uninfected RBCs with the Best Signal to Background. Several different fluorescent DNA dyes were tested at the same concentration (1:1000) in RIPA buffer for

their ability to discriminate between infected and uninfected RBCs. Ethidium iodide (EtBr) and PI were the only dyes tested that gave a statistically significance difference when analyzed by a Welch's t Test. PI had a higher signal to background over EtBr, making it a better fluorescent dye for the needs herein. SYBR Green (SG) did not produce a statistically significance difference and had the opposite results from the expected. Experiment was carried out by collecting pure blood from an infected culture, making a 5% hematocrit solution in cDMEM, and aliquoting 50µL into a well of a black, clear bottom 384-well plate alongside 50µL of uninfected 5% hematocrit solution in cDMEM. Each fluorescent DNA dye was diluted 1:1000 in RIPA buffer before 50µL was added in duplicates to the infected and uninfected RBCs, the plate incubated for 1 hour, and plate read. Abbreviations and excitations/emissions: AO = Acridine Orange (500/526); EtBr = Ethidium Bromide (301/603); Hoescht = Hoescht 33528 (352/458); PI = Propidium Iodide (535/617); SG = SYBR Green (485/530).

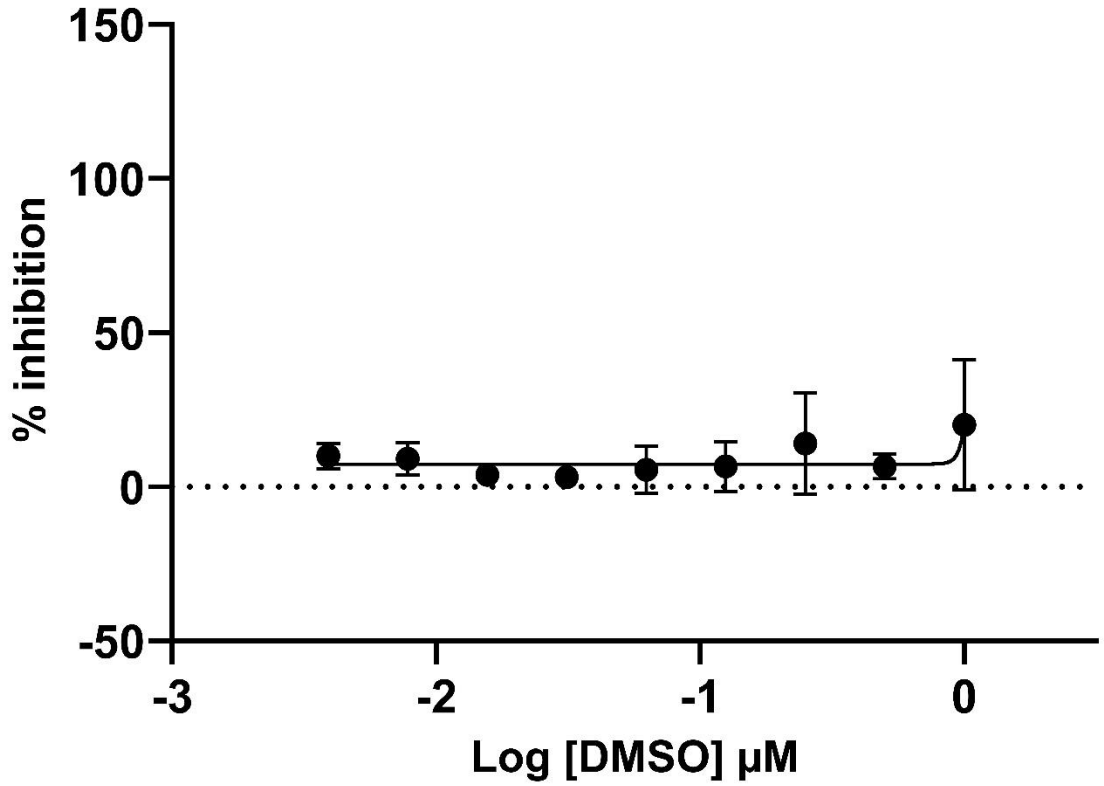

Figure 3. High Concentrations (≥1%) of Dimethyl Sulfoxide (DMSO) Inhibit Babesia duncani Growth. Dose response curve of DMSO shows that Babesia duncani growth is inhibited by ≥1% (or ≥0 µM) final DMSO in wells. 1% is four times more than the final concentration typically present in wells, meaning there is a high safety margin. This is an important understanding for the compound screening assay as DMSO is the compound vehicle, and some compounds are less soluble in it than others, meaning the compound is less concentrated at its starting point in the dilution series and more DMSO is present. Dose response curve was generated as described in compound testing in Chapter Four.

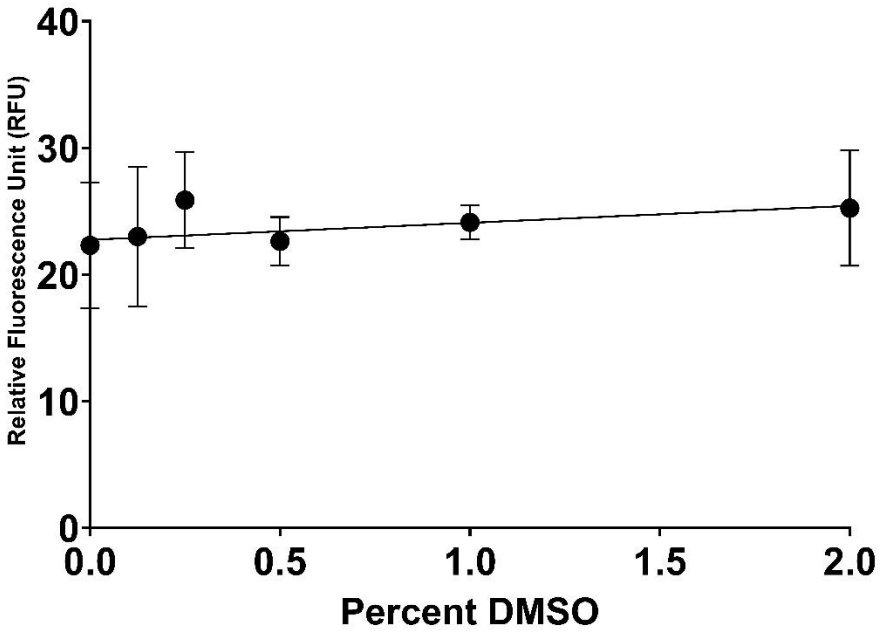

Figure 4. Dimethyl Sulfoxide (DMSO) Concentration does not Impact Assay Readout as Relative Fluorescence Unit (RFU) is not Correlated with Percent DMSO. A simple linear regression was performed and the r^2 was 0.03, indicating there is no correlation between RFU and percent DMSO. This means that the DMSO is killing Babesia duncani at ≥1% final concentration. Experiments were performed by serially diluting cDMEM with DMSO, transferring 25µL in triplicates to a white, clear bottom 384-well plate, adding 25µL of cDMEM followed by 0.6% Triton-X, 22.5µM Propidium Iodide (PI) in 1x Phophate Buffer Solution, no Ca2+ or Mg2+ (PBS) and reading immediately as previously described in Chapter Four with the sensitivity changed to 50.