ABSTRACT

Analysis of the Microfilariae-Specific Transcriptome of *Brugia pahangi* During Mosquito Infection

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Development of a transmission-blocking vaccine may provide cost-effective means of preventing the spread of lymphatic filariasis. Here, vaccine candidates were derived from the transcriptome profiles of two separate developmental life stages of *B. pahangi*: microfilarial and third-stage larval. Analysis of these differential transcripts provided a detailed overview of expression profiles unique to each developmental stage. Differentially expressed transcripts were scored before the 14 highest ranked transcripts were validated via qRT-PCR. Seven candidate genes were identified as potential targets for transmission-blocking vaccines; two types of Serpins, two types of Cathepsin, Hsp70, Ubiquitin, and Chitinase. Gene selection was limited to secretory or transmembrane proteins in order to increase the proficiency of transmission-blocking vaccine candidates. Finally, we discussed the potential role of each candidate gene in *B. pahangi* during the midgut infection stage in the mosquito *Culex pipiens*.

Analysis of the Microfilariae-Specific Transcriptome of *Brugia pahangi* During Mosquito Infection

by

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LIST OF ABBREVIATIONS

TBV	Transmission Blocking Vaccine
DEC	Diethylcaramazide
IVM	Ivermectin
FPKM	Fragments Per Kilobase of exon per Million fragments mapped
B. pahangi	*Brugia pahangi*
Cx. pipiens	*Culex pipiens*
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas9	CRISPR-associated protein 9
ZFN	Zinc-figner nucleases
TALEN	Transcription activator-like effector nuclease
PAM	Protospacer Adjacent Motif

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DEDICATION

To my mother Ms. Antoinette M. Favorite

for all the love you have given me

I dedicate my science to you

EPIGRAPH

Science should be used for the betterment of human kind. – Dr. Thomas Light

CHAPTER ONE

Introduction

Filarial nematode infections are a major cause of long-term disabilities, economic hardships and social stigma. Lymphatic filariasis, caused by *Wuchereia bancrofiti*, *Brugia malayi*, *Brugia timori*, and *Brugia pahangi* infections, has become the target of elimination by mass drug administration (MDA) (WHO 2015, Mak 1987, Moreno et al. 2008). However, recently filarial netmatodes are developing drug resistance in the endemic regions where MDA programs have been established. This necessiates novel approches to disease treatment and prevention related to lymphatic fialriasis. The life cycle of filarial nematodes begins when female mosquitoes take a blood meal from an infected human, ingesting the microfilariae filarial nematodes. Microfilariae, ingested during the blood meal, must subsequently survive digestive enzymes and pass through the wall of the midgut into the thoracic tissue where they develop into third-stage larvae. Infections in humans start when an infected female mosquito takes a second blood meal releasing third-stage larvae into the host. Development of a transmission-blocking vaccination may provide an alternative and a cost-effective mean of preventing the spread of diseases such as lymphatic filariasis. Microfilariae development seems to be the logical target of a transmission-blocking vaccine prevent mosquitoes from up taking a Brugia infection.

B. pahangi nematodes were once thought to solely cause infections in house cats, recently it was discovered that *B. pahangi* nematodes also cause infections in humans presenting with lymphatic filarisis sympotoms (Tan et al 2011). This new report suggest *B. pahangi* infections may have been under reported in humans. In chapter two we study the

differential expression of *B. pahangi* microfilariae and third-stage larvae in the mosquito *Culex pipiens*. We aim to find candidate targets with the intent to develop at least one of them into a successful transmission-blocking vaccine limiting *B. pahangi* infections in humans.

The major vector for lymphatic filariasis is the *Culex* complex mosquitoes (Curtis et al 2002, Mak 1987). Recent years has brought more attention to diseases spread by *Culex* mosquitoes. The impact of mosquito borne diseases calls for a method to eliminate the spread of diseases from mosquitoes. In the past the use of dichlorodiphenyltrichloroethane (DDT) and other insecticides were the main way of controlling mosquito populations. However, the ability for mosquitoes to adapt has insecticides has reduces the impact of insecticides on mosquito population over time. Recent advances in genetic approaches has increased our understanding of the life cycle and molecular processes of mosquitoes. As our understanding of these diseases vectors increase the need for techniques to regulate the spread of mosquito borne diseases becomes increasingly more in demand.

In chapter three we develop a protocol to use the CRISPR/Cas9 system in vitro as a primary screen of our in lab synthesized CRISPR/Cas9 system. Chapter three also details the development of a protocol using the CRISPR/Cas9 system in *Cx. pipiens*. Many mosquito complexes have already been studied using the CRISPR/Cas9 system (Kistler et al 2015). The differences in diseases carried by these mosquitoes make it important to develop effective gene editing tools in each mosquito complex.

CHAPTER TWO

Differentially Expressed Transcriptome Analysis Reveals Candidate Antigens for a Transmission-Blocking Vaccine of *Brugia pahangi* Infection.

Abstract

Lymphatic filariasis is endemic to many tropical and subtropical regions worldwide. The cause of lymphatic filariasis in humans is infection of the lymphatic system by *Brugia* nematodes. Even with mass drug administration efforts lymphatic filariasis is still a major disease plaguing endemic regions. Development of a transmission-blocking vaccine provides an alternative approach to preventing *Brugia* parasitic infections at a low cost and high efficacy. By analyzing the transcriptome of *Brugia pahangi* microfilariae and third-stage larvae we identified the genes related in limiting factor of midgut infection in the mosquito. These genes can thus serve as candidate targets for transcription-blocking vaccines.

Vaccine candidates were derived from the transcriptome sequence used to profile two developmental life stages of *B. pahangi*: microfilariae and third-stage larvae. Analysis of these two life-stage transcriptomes provided a detailed overview of expression that differentiates these two lifecycle stages. Using scoring criteria, differentially expressed transcripts were ranked. Quantitative analysis of the 14 highest ranked transcripts was determined by qrt-PCR.

We determined seven candidate genes to be used as targets for transmission-blocking vaccines; two types of Serpins, two types of Cathepsin, Hsp70, Ubiquitin, and Chitinase. To increase proficiency of transmission-blocking vaccine candidates' secretory

or transmembrane proteins were exclusively selected. We discussed the potential role of each gene in *B. pahangi* during the midgut infection stage in the mosquito *Culex pipiens*.

Introduction

Worldwide it is estimated that 1.1 billion people in 73 countries living in subtropical or tropical regions are at risk of infection by filarial nematodes [1]. Tropical filarial nematodes *Wuchereria bancrofiti*, *Brugia malayi*, *Brugia pahangi* and *Brugia timori* are known to cause lymphatic filariasis, commonly referred to as elephantiasis [2,3]. Spread to humans by mosquito vectors, the lymphatic filariasis afflicted by these nematodes often result in severe disfigurement and disability. The development of *Brugia* nematodes in the lymphatic system is the cause symptoms ranging from swollen lymph nodes, hydrocele, to complete enlargement of body parts and skin exfoliation. These symptoms may cause long-term physical problems and are often accompanied by economic and social hardships associated with disfigurement [4].

Nematodes that cause lymphatic filariasis such as *Brugia* have a dualistic host life cycle, living inside human and mosquito host. The start of infection in humans begins when an infected female mosquito bites them to take a blood meal. At this moment a pathway is formed allowing the third-stage larvae to enter and infect the human. Once inside the human host, third-stage larvae will make their way into the lymphatic system where they will continue their development. Over the next two weeks third-stage larvae will molt and develop into fourth stage larvae. Within a year, fourth-stage larvae will develop into fully mature adults, and impregnanted adult females will develop embyros in utero until they become microfilariae. Thoundsands of microfilariae are consequently released into the lymphatic system daily, and will travel from the lymphatic system in the circulatory

systems and spread throughout the body. The microfilariae will continue to circulate throughout the body until another female mosquito takes a blood meal from the infected human. Ingested microfilariae will enter into the gut of the mosquito where they must survive digestive enzymes before crossing the peritrophic matrix and the wall of the midgut into the hemocele. Once they reach the thoracic muscle tissue of the mosquito the microfilariae will molt twice as they develop into thrid-stage larvae. *Brugia* nematodes will then halt development as they migrate to the mosquito salivary gland. At this point *Brugia* nematodes are ready to begin a new cycle of infection in a mammalian host [3].

Health organizations are currently waging a war against diseases caused my *Brugia spp*. nematodes [1]. Their current efforts include the use of diethylcaramazide (DEC) and ivermectin (IVM) as anti-microfilarial agents [5-8]. However, they require multiple doses to have an optimal effect against microfilariae. Furthermore, this appaorch does not prevent the transmission of *Brugia* nematodes and in some cases fail to eliminate the microfilarae completely despite extensive drug therapy [9,10]. It is likely that due to continuous DEC and IVM therapy in endemic regions, *Brugia* microfilarae are developing resistance to both drugs necessiating novel approches to disease treatment and prevention. Transmission-blocking vaccination (TBV) has shown promise as an alternative to conventional vaccination approaches [11-15].

This new approach is a cost-effective means of controling the spread of lymphatic filarisis long term. The goal of TBV is to prevent the spread of lymphatic filarisis by introducing antibodies that prevent the *Brugia* from maturing inside the mosquito after a blood meal is taken from a vaccinated host. Unfortunately, this approach neither prevents vaccinated individuals from contracting the disease nor cure the symptoms. It does,

however, decrease the rate of infection by preventing mosquitoes that fed on infected host from continuing to transmit *Brugia* to new host. Another benefit of TBV is that there is no pre-vaccination IgE effect. This is due to the fact that antigens produced are not from the parasite during human infection stage.

A recent study has supported the idea that *B. pahangi*, a filarial nematode commonly found in house cats, may cause infections in humans and present symptoms mirroring lymphatic filarisis [16]. *B. pahangi* infections may have been under reported in humans. With the completion of the *B. pahangi* draft genome, advancements in whole genome expression profiling can be used to be characterization their development [17]. Here we characterize the transcriptional differences between microfilariae and third-stage larvae of *B. pahangi* in the mosquito *Culex pipiens*. It is our goal by doing so to find a list of targets that we can use to develop transmission-blocking vaccine for *B. pahangi* infections in humans.

Materials and Methods

RNA Sample Preparation from Brugia pahangi

B. pahangi microfilariae were obtained from the peritoneal cavities of patently infected dark-clawed Mongolian gerbils (*Meriones uguiculatus*) by peritoneal flush with pre-warmed RPMI media (Fisher Scientific, Piscataway, NJ). Microfilariae were then purified by centrifugation through Ficoll-PaqueH lymphocyte isolation media (Amersham Pharmacia Biotech, Piscataway, NJ), and washed in PBS three times. For collection of *B. pahangi* third-stage larvae, 12 days following an infected blood meal, mosquitoes were crushed in RPMI and L3 collected in a Baermann apparatus. The collection of the microfilariae and third-stage larvae samples and preparation of total RNAs was conducted

in the NIAID-NIH Filariasis Research Reagent Resource Center at University of Georgia, Athens, GA and Smith College, Northampton, MA.

Library Preparation for Illumina RNA Sequencing

Library preparations were completed using TruSeq mRNA library construction method. Molecules of mRNA were purified twice using poly-T oligo-attached magnetic beads. As part of the second elution, poly-A RNA was fragmented and primed for cDNA synthesis. The first strand of cDNA was synthesized by reverse transcribing the fragmented RNA using random hexamer primers. A second strand was generated by removing the RNA template and synthesizing a second strand to generate (ds) cDNA. cDNA was serperated from the 2nd strand reaction mix using Ampure XP beads. The overhang from fragmentation was turned into blunt ends using end repair. A single adenyation was added to the 3' ends to prevent both strands from ligating together during the adapter ligation reaction. Multiple indexing adapters were then ligated to the ends of the ds cDNA and selectively enriched by PCR.

Primary Processing of Illumina RNA-Seq Reads

The millions of unique clusters were then loaded onto an Illumina HiSeq 2000 platform for automated extension and imaging cycles, using a Solexa Sequencing-by-synthesis method where all four nucleotides reversible fluorophore and termination properties. Sequences were extend one base at a time.

Mapping of RNA-Seq Reads Using TopHat and Cufflinks

Reads were then processed and aligned to the Sanger *B. pahangi* draft genome using TopHat v1.0.12 [18]. TopHat incorporated the Bowtie v0.11.3 algorithm to perform

the alignment. TopHat initially removed a portion of reads based on quality information accompanying each read, then mapped reads to the reference genome. TopHat allowed multiple alignments per read (up to 40 by default) and a maximum of 2 mismatches when mapping reads to the reference. The mapping results were then used to identify "islands" of expression, which were interpreted as potential exons. TopHat builds a database of potential splice junctions and confirms these by comparing the previously unmapped reads against the database of putative junctions. Default parameters for TopHat are used.

The aligned read files were processed by Cufflinks v0.8.0 [19]. Reads were assembled into transcripts, their abundance estimated and tests for differential expression and regulation between the tissue samples were performed. Cufflinks did not make use of existing gene annotations during assembly of transcripts, but rather constructed a minimum set of transcripts that best described the reads in the dataset. This approach allowed Cufflinks to identify alternative transcription and splicing that were not described by pre-existing gene models [19] Cufflinks used the normalized RNA-Seq fragment counts to measure the relative abundances of transcripts. The unit of measurement was Fragments Per Kilobase of exon per Million fragments mapped (FPKM). Confidence intervals for FPKM estimates were calculated using a Bayesian inference method [20].

Comparison to Reference Annotation and Differential Expression Testing

Once all short read sequences are assembled with Cufflinks, the output .GTF files are sent to Cuffcompare along with a reference .GTF annotation file downloaded from the Sanger database. This classifies each transcript as known or novel. The classification also describes the nature of the match to the reference gene annotation by way of a code letter. These were useful for selecting novel isoforms from the analysis. Cuffcompare produced

a combined .GTF file which was passed to Cuffdiff along with the original alignment (.SAM) files produced by TopHat. Cuffdiff then re-estimated the abundance of transcripts listed in the .GTF file using alignments from the .SAM file, and concurrently tested for differential expression. The expression testing was done at the level of transcripts, primary transcripts and genes.

Gene Ontology

DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resource v 6.7 was utilized to cluster significant changes in gene expression [21]. Genes were then classified by biological process and molecular function via the GO (Gene Ontology). Manual Gene Ontology search was performed on uniprot.org (http://www.uniprot.org).

qRT-PCR Validation of Differential Expression

To validate transcript level differences of target genes in microfilariae and third-stage larvae, total RNA samples from microfilariae and third-stage larvae were provided by Filariasis Research Reagent Resource Center and cDNAs was reverse-transcribed via SuperScript III as described by manufacturer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). qRT-PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Briefly, standard curves were generated for each transcript tested using tenfold serial dilutions of cDNAs which included each target gene, ranging from 100 to 0.01 pg per reaction. All reactions were performed in triplicate in a total volume of 20 µl containing 10 µl of SYBR Green PCR Master Mix and 300 nmol of each primer at the following conditions: 95 °C for 10 min followed by 45 cycles of

denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Putative U2 auxiliary factor (U2) (BPAG_0001183401) was used as an internal control, which was also used as an internal control of qRT-PCR analysis in *B. malayi* [22]. Statistical significance of differences in the expression of individual genes was determined by using a Student's t-test between the relative transcript values, and a P-value less than 0.05 was considered to be a significant transcript level change. Candidate genes and primer information can be found in Table 2.1.

Results

Transcriptome Analysis by Deep Sequencing

The transcriptome of *B. pahangi* was analyzed at two life development stages: microfilariae and third-stage larvae by RNA-Seq. Sequencing was done by HiSeq 2000 generated 34 million processed reads (total 3.4 Gb) for *B. pahangi* microfilaria. HiSeq 2000 sequencing generated 26 million processed reads (total 2.7 Gb) for *B. pahangi* third-stage larvae.

Transcripts were aligned to the draft genome from the whole genome shotgun (WGS) of *B. pahangi* (GenBank: PRJNA263436) [17]. A sequence alignment was able to align approximately 50.7 % of the transcripts of *B. pahangi* microfilaria to the reference genome. For third-stage larvae sequence alignment was able to align 60.1 % to the draft genome of *B. pahangi*. However, to determine the quality of the alignment multiple mapping sites and low quality reads were detected by TopHat. 15.2% % of transcripts for

Table 2.1. List of candidate genes and primers used for qRT-PCR.

Gene Name	Gene ID	Primer	Sequences
Chitinase	BPAG_001455501	For	AGTTGGGAGAAGAACGTCGA
		Rev	TCCATCCCTGAGCGTACATC
Serpin-1	BPAG_0000262701	For	TGCAATCATCACCGCATTACT
		Rev	CTCCTTCGTTTTGCCATCCG
Cathepsin-1	BPAG_0000236501	For	TTGCCGCCTAACAAAGAAGG
		Rev	CCGCACATAAATACCCCAGC
Cathepsin-2	BPAG_0000291501	For	TTACCACCATCACCACCACC
		Rev	TGCTGAATCAAGTATTGGCTGT
Hsp-70	BPAG_0000788401	For	GGACGAATTTCCGGTGATCA
		Rev	TTCCTGAACCGAAAGCTTGC
Thioredoxin	BPAG_0001116901	For	CAAGCCGAGACCAGTGAAAG
		Rev	GTGCGACTGTACCGAATCAC
Papain	BPAG_0000291601	For	AGCGATTGGACGGAAGAAGA
		Rev	AAACACACACCACAACGACC
Serpin-2	BPAG_0000329501	For	GGCAGTACTCGGTGGTAGTT
		Rev	TCACGCTCAGTTTCACCTCT
Chaperonin	BPAG_0001198501	For	TGGAGCTGTCGAGGTTGAAT
		Rev	GGCGTTTCTTTGACCGTCTT
Ubiquinol	BPAG_0001259901	For	TTAGCCCCGAATGAGCAAAA
		Rev	TCCGGTAAGCTTCGTAGTTCA
Hsp-90	BPAG_0000139801	For	GCACTGCGGGATTCTTCTAC
		Rev	CTGCGGATCTTCAAGCGAAA
Alpha Amylase	BPAG_0000901901	For	TCAAATCAGCAAAAGCGGGT
		Rev	TGGAAGCCATCAACTCCCAT
HP-1	BPAG_0001318201	For	TTTGGCAAGAGATCAGCACC
		Rev	CAATCATATTCCCAGATGCCGG
Ubiquitin	BPAG_0000881201	For	TCTTGAGATTGCGAGGTGGT
		Rev	CTAGCCATGAAAACGCCTCC

transcripts having a 2 fold (Log_2) increase in relative transcription in the microfilaria were taken into consideration for antigen candidates (Table 2.2).

Gene Ontology Analysis

A Gene ontology analysis was carried out on the 64 transcripts having a 2 fold (Log_2) upregulation in microfilaria when compared to third-stage larvae using the DAVID (Database for Annotation and Visualization and Integrated Discovery) tool v 6.7 [23-24]. DAVID analysis determined 22 transcripts mapped to 11 different known biological

processes, and 32 transcripts mapped to 17 different known molecular functions. Transcripts were further analyzed manually to determine the gene ontologies of transcripts that could not be determined by DAVID Analysis. A manual search added 8 additional biological processes and 9 additional molecular functions. In total, the gene ontology analysis determined the biological process of 30 transcripts and the molecular function of 41 transcripts.

Structural constituents of the ribosome (GO:0003735) and nucleotide binding (GO:0000166) were two of main molecular functions listed from the GO analysis. Structural constituents of the ribosome represented 12 of 64 of transcripts and nucleotide binding represented 7 of 64 of transcripts (Figure 2.1A). Other molecular functions revealed by GO analysis were: Cysteine-Type Peptidase Activity (GO:0008234) 4 of 64, Calcium ion binding (GO:0005509) 3 of 64, Zinc ion binding (GO:0008270) 2 of 64, Ion Binding (GO:00043167) 2 of 64. Molecular functions Alpha, alpha-trehalase activity (GO: 0004555), ATP binding (GO: 0005524), Chitinase Activity (GO:0004568), Enzyme Inhibitor Activity (GO:0004857), Peptidyl-prolyl cis-trans isomerase activity (GO:0003755), Peroxidase activity (GO:0004601), Protein Binding (GO:0005515), Serine-Type Endopeptidase Inhibitor Activity (GO:0004555), Structural Molecule Activity (GO:0005198), Translation Elongation Factor Activity (GO:0003746), Ubiquinol-Cytochrome-C Reductase activity (GO:0008121) each only had one transcript listed. The molecular functions of 23 transcripts remain undetermined. Translation (GO:0006412), protein folding (GO:0006457), and proteolysis (GO:0006508) were the top three biological processes listed by the GO analysis (Figure 2.1B). Each biological process had 13 of 64, 5 of 64, and 4 of 64 transcripts of each respective category. Other

biological functions each only had 1 transcript listed and included: Cellular Homeostasis (GO:0019725), Chitin Catabolism (GO:0006032), DNA Metabolism (GO:0006259), Positive Regulation of MAPK Cascade (GO:0043410), Protein Amino Acid Phosphorylation (GO:0016310), Protein Localization (GO:0008104), Protein Processing (GO:0016485), Trehalose Metabolism (GO:0005991). The biological process of 34 transcripts remain undetermined.

Candidate Selection

Each transcript was scored in 6 categories to determine the likelihood of producing an antigen: Abundance in Microfilaria (0-5), Function related to survival (0-3), Signal peptide (0-3), Transmembrane (0-3), Protective homologue (0-3), and Expression feasibility (0-3). The final scores ranged between 20-0. (Table 2.2). To validate the results of RNA-Seq, transcription levels of the 14 highest scoring transcripts were selected to be quantified by qRT-PCR. By using qRT-PCR to validate Chitinase, Serpin-1, Serpin-2, Cathepin-1, Cathepin-2, Hsp-70, and Ubiquitin we were able support the accuracy of the RNA-Seq data (Figure 2.2).

Discussion

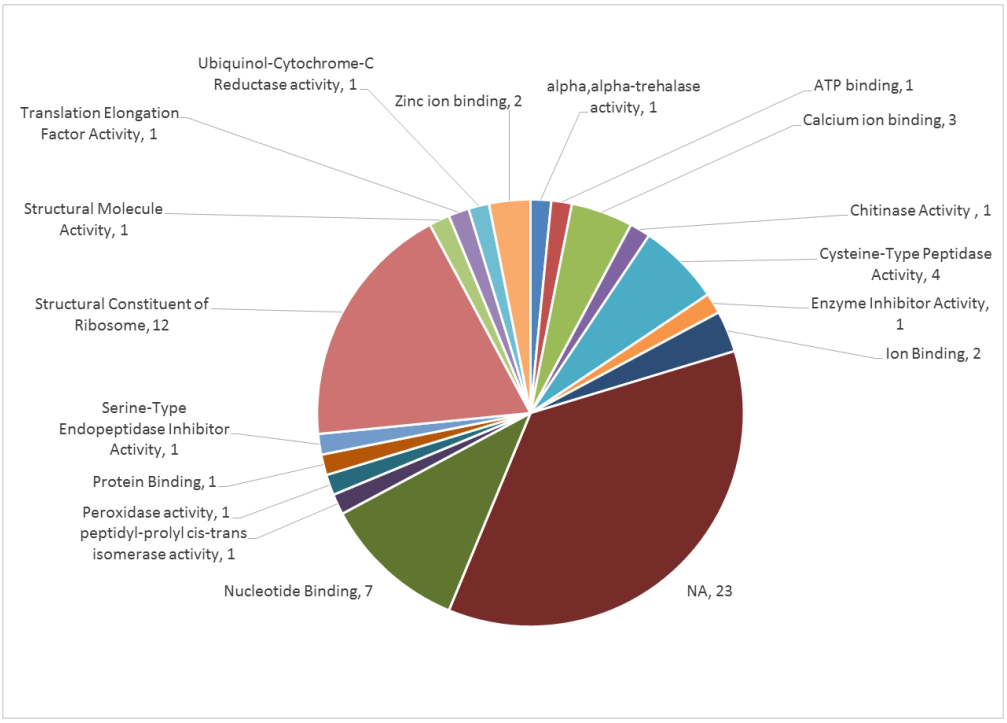
There is a growing need for development of a vaccine against *B. pahangi* parasites in many developing countries. Higher treatment costs associated with manufacturing, efficacy trials and distribution may inhibit the availability of medicines in these regions. A new vaccine that is low cost and greatly prevents the spread *of B. pahangi* parasites may come best in the form of a transmission-blocking vaccine. The role of these transmission-blocking vaccines is to prevent the spread of filarial nematodes during the vector infection

stages. In this study we used *B. pahangi* and its vector *Cx. pipiens* as a model system to elucidate the molecular interaction during midgut infection stage in the mosquito as our target for transmission blocking. The newly assembled genome of *B. pahangi* has been recently published, and was used to perform high quality and inexpensive genome-wide transcriptome analysis of *B. pahangi* [17]. In determining candidates for an effective transmission-blocking vaccine against the midgut infection of *B. pahangi*, it is important to make sure each candidate is related to a key function in *B. pahangi* development or infection through the midgut of its vector *Cx. pipiens*.

In *Anopheles sp*. antigens from the midgut have been shown to have greater likelihood to become suitable candidates for transmission-blocking vaccine components [25-28]. The migration through the midgut by *B. pahangi* is a critical developmental stage. The first stage of *B. pahangi* infection starts when a blood meal containing microfilariae is ingested by a *Cx. pipiens* adult females and reaches the midgut. Before *B. pahangi* can pass through the wall of the midgut they must first pass through the peritropic matrix, which is made of chitin and begins its formation when a blood meal is detected inside the body of the mosquito. Once formed, the peritropic matrix surrounds the blood meal and acts as the first barrier preventing microfilariae from reaching the midgut wall [29,30]. We have successfully validated the up regulation of chitinase (BPAG_001455501) in *B. pahangi* microfilariae. It may be the role of this chitinase to be secreted by the microfilairae and assimilate to the peritropic matrix or midgut epithelium to allow them to easily penetrate to the midgut [31,32]. Furthermore, microfilariae penetration of the midgut can be enhanced in the presence of N-acetylglucosamine and chitinase [33].

Microfilariae are surrounded by what is known as the microfilarial sheath, comprised of the remains of the eggshell that once coated the developing microfilariae. [34]. The microfilarial sheath plays a crucial role in the interaction between microfilariae and the peritropic matrix in the mosquito midgut. Microfilariae remove their sheaths after successful migration from the midgut into the hemolymph of the infected mosquito. [35-36]. The microfilarial sheath is composed of many molecules including chitin, proteoglycans, phospholipids, acidic glycosaminoglycans, β1, 4-linked N-acetylglucosamine and matrix proteins [35, 37-42]. As microfilariae mature the sheath is modified and exhibit diminished ability to unsheathe [35, 37, 43, 44]. Alterations to β1, 4-linked N-acetylglucosamine can be made by different types of chitin synthesis inhibitors [42]. It is not yet known whether chitinase's (BPAG_001455501) role is to interact with the peritropic matrix or the microfilarial sheath and its function during these biological process remain elusive.

The cuticle is a structure, below the sheath, made up of disulfide-linked collagens molecules and lipids. It has also been shown that there is a link between alkaline phosphatases and calcium-activated ATPase activities with the cuticle of the microfilariae [41, 45, 46]. As microfilariae develop into first stage larvae, they gradually change the composition of their cuticle proteins, lipids, and carbohydrates [40,47-49]. Highly regulated processes such as molting involve the removal of the current cuticle allowing for the synthesis and remodeling of the new cuticle [50, 51]. In addition, we validated the up regulation of two cathepsin L-like cysteine proteases (BPAG_0000236501, BPAG_0000291501) and two serpins (BPAG_0000262701, BPAG_0000329501) in microfilariae. It is then likely these genes encoding serpins and cathepsin L-like proteases



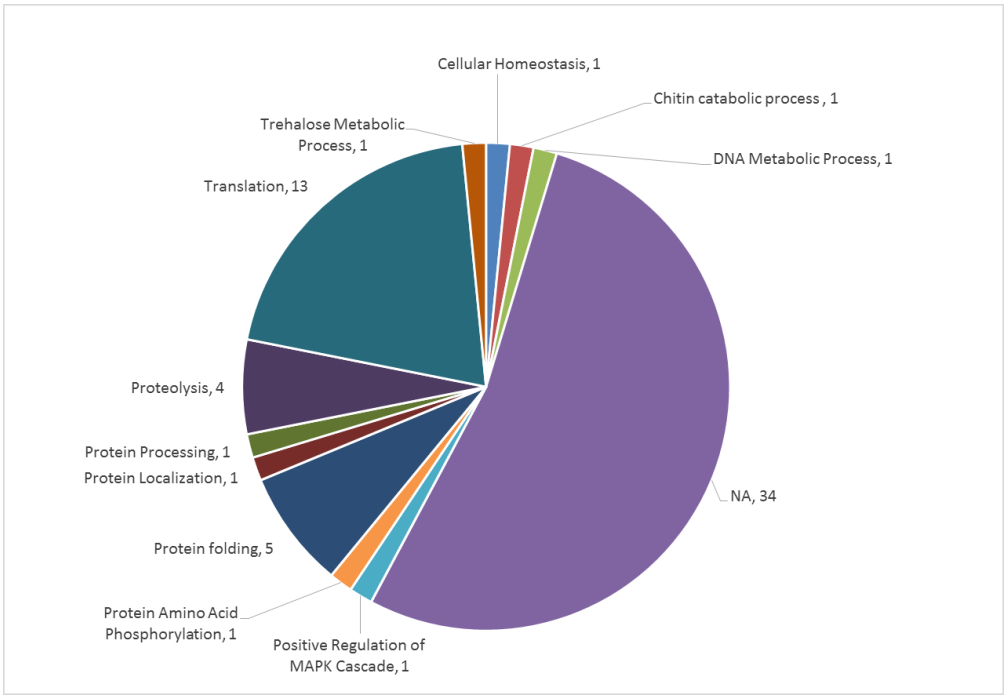


Fig 2.1. Gene ontologies of $\log_2$ fold change transcripts in microfilaria. A. Molecular Function, B. Biological Process; NA = not yet assigned in gene ontologies; each number indicates total transcript number assigned in the gene ontology category.

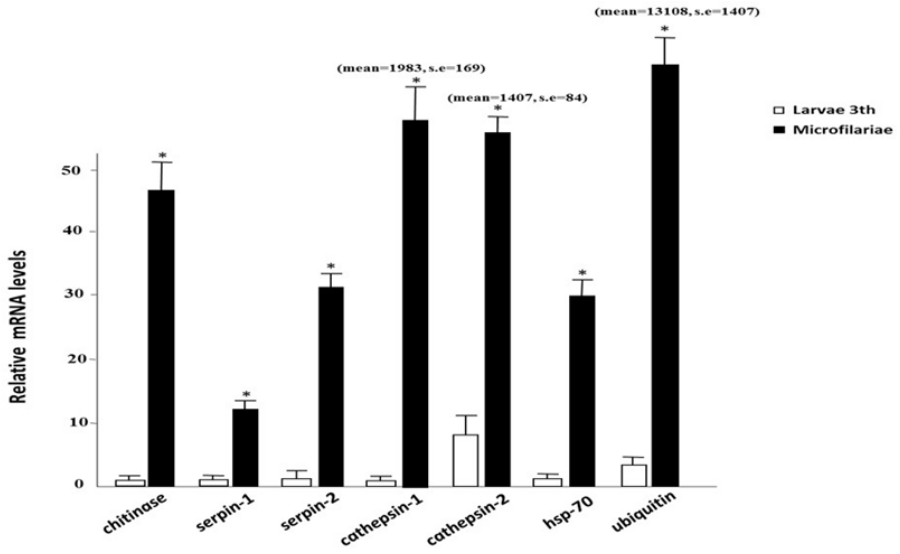


Fig 2.2. qRT-PCR validation of 7 possible antigen candidates. 7 transcripts among 14 candidate genes were validated in which microfilaria transcription levels were significantly higher compared to those present in L3 larva.

are associated with processes that are unique to microfilariae and their cuticle remodeling processes.

With both the microfilarial sheath and cuticle undergoing such heavy protein modification it is important that the microfilariae have a mechanism of protein removal. The up regulation of ubiquitin (BPAG_0000881201) in microfilariae suggests an association with the regulation of proteins specific to the microfilariae. The importance of ubiquitin in the regulation of proteins is well documented as is its nature as a pervasively distributed protein that targets a multitude of proteins for modification, degradation, and cellular relocation determining a specific process regulated by ubiquitin.

Heat shock proteins 70-kDa (Hsp70) are known to help regulate many processes of protein folding. Hsp70 has two domain regions, one at each end of the protein. On the N-terminal end Hsp70 has a nucleotide-binding domain and on the C-terminal end a substrate-binding domain [52]. Interestingly, GO analysis of top up-regulated transcripts show

Table 2.2. Top 14 Antigens ranking for transmission vaccine targets from *Brugia pahangi* microfilaria.

Bp ID	Experimental Name	MF/L2_3	Abundance in MF	Function related to survival	Signal peptide	Transmembrane TM	Protective homologue	Expression feasibility	Final score
BPAG_0000236501	Cathepsin-1	5.01	3	3	0	3	3	3	15
BPAG_0000262701	Serpin-1	7.33	3	3	0	3	3	3	15
BPAG_001455501	Chitinase	7.77	4	3	3	0	3	2	15
BPAG_0000291501	Cathepsin-2	6.65	3	3	3	0	3	2	14
BPAG_0000788401	Hsp-70	3.53	2	3	0	3	3	3	14
BPAG_0001116901	Thioredoxin	2.97	2	3	3	0	3	3	14
BPAG_0000291601	Papain	5	3	3	3	0	3	1	13
BPAG_0001198501	Chaperonin	2.65	2	3	0	3	3	2	13
BPAG_0000329501	Serpin-2	4.32	2	3	3	0	3	2	13
BPAG_0001259901	Ubiquinol	2.57	2	3	0	3	0	3	11
BPAG_0000139801	Hsp-90	2.52	2	3	0	3	0	1	9
BPAG_0000901901	Amylase	2.12	1	3	0	3	0	2	9
BPAG_0001318201	HP-2	4.51	2	0	3	0	0	3	8
BPAG_0000881201	Ubiquitin	4.56	2	0	3	0	0	2	7
BPAG_0000888801	RNA-Binding	4.3	2	0	0	3	0	2	7

transcripts that are involved in nucleotide binding and seven transcripts with a role in protein processing, localization, or folding. It may very well be the case that these transcripts have a role with Hsp70. In a recent study, a small molecule inhibitor of Hsp70 ATPase activity was shown to increase production of antibodies against immunized antigens in mice [53]. The potential of targeting Hsp70 with other small molecules may as serve as a candidate for transmission-blocking vaccine.

In this study we provide a list of candidate genes to be studied as a transmission-blocking vaccine. We used RNA deep sequencing and qRT-PCR to measure and validate differences in the transcriptome of *B. pahangi* microfilariae and 3rd stage larvae. As the primary purpose of this study was to find candidates for a transmission-blocking vaccine, we increase our rigor by only taking transcripts with a 2x fold increase in transcription for microfilariae when compared to L3 larvae. These selected transcripts were validated by qRT-PCR and showed coherent profiles with the RNA-seq results from RNA samples of the microfilariae and L3 of *B. pahangi*. Feasibility as a transmission-blocking vaccine was improved by selecting genes that are either signal proteins (BPAG_001455501, BPAG_0000291501, BPAG_0000329501, and BPAG_0000881201) or transmembrane proteins (BPAG_0000236501, BPAG_0000262701, BPAG_0000788401). To further determine the ability of these genes for a transmission blocking vaccine candidates they will be expressed in two eukaryotic expression systems, the yeast expression system and the baculovirus/insect cell system. The expressed and purified recombinant proteins will be characterized for identity, conformation (folding), yield, purity, and functional activity. These high-quality recombinant proteins will then be used for pre-clinical trial using *B. pahangi* and its vector model for the future proposed studies.

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CHAPTER THREE

Development of the CRISPR/Cas9 System for Targeted Gene Disruption in *Culex pipiens*

Abstract

Clusters of regularly interspaced short palindromic repeats (CRISPR) are part of the adaptive immune systems of bacteria. With the help of CRISPR-associated protein 9 (Cas9), CRISPR RNAs recognize foreign DNA and target them for cleavage by the Cas9 nuclease. The recent discovery of CRISPR-Cas9 system has given rise to advances in whole genome editing techniques. PCR was used to synthesize the CRISPR DNA template followed by in vitro transcription to generate active CRISPR sgRNA. PCR was also used to generate an amplicon to be targeted for CRISPR-Cas9 digest. Here we develop a protocol to synthesize the necessary components of the CRISPR-Cas9 system. The in vitro test enables to determine efficacy of our synthesis and determine that each component is enzymatically active before use for targeted gene disruption in germline.

Introduction

In the United State, *Culex* complex mosquitoes are the major vector for West Nile virus [1-7] as well as Lymphatic filariasis [8, 9]. During the spring and summer *Culex sp.* are primarily avian feeders and switch to mammalian prey during the fall [10]. The impact of mosquito borne diseases calls for a method eliminate the spread of diseases from mosquitoes or to control mosquito populations reducing the probability of infected mosquitoes spreading diseases. Genetic approaches has increased our understanding of how many of these diseases are transmitted. As our understanding of these diseases

increase the need for techniques to regulate the spread of mosquito borne diseases becomes increasingly more in demand.

In the recent years there has been a development of a high number of genome editing techniques including zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and meganucleases. These techniques rely combining the catalytic domain of endonucleases together with a string of DNA binding proteins to create targeted double strain breaks (DSB) at a desired loci. Each of these techniques has been used in a variety of different organisms to preform genome editing. Meganucleases are engineered restriction enzymes typically have extended DNA recognition sequences between 14–40 nucleotides long. However the construction of meganucleases has proven to be challenging for biologist due to the DNA recognition domain and cleavage domain resting in a single domain [11, 12].

Unlike mega nucleases, ZFNs and TALENs are engineered fusion proteins artificial fusion proteins composed of an engineered DNA binding domain fused to a nonspecific nuclease domain from FokI. ZFN and TALEN DNA recognition domains and be ligated together into long arrays binding to extended DNA sequences. Much like meganucleases, the construction of ZFN is difficult for many laboratories to achieve due to the context-dependent effects between the individual finger domains in a complex [13]. Even with a great number of available protocols designed to simplify the challenge of creating ZFNs this technique has not become widely implemented [14-20]. In contrast to both meganucleases and ZFN, TALENs are easier to design and the assembly of DNA sequences coding for the highly conserved TALEN repeat domain can be acquired using a

multitude of techniques [21]. The ease of designing TALENs compared to meganucleases and ZFNs has opened them up for used by a number of scientists.

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Cas9 (CRISPR-associated protein 9) system used in a part of the adaptive immune system of bacteria and archaea to protect against viruses and plasmids. The type II CRISPR system takes any foreign DNA and incorporates it into the CRISPR repeat sequence found in the bacterium's genome. CRISPR RNA (crRNA) is formed from the CRISPR repeat sequences include the sequence of the foreign DNA. A crRNA hybridizes to a trans activating CRISPR RNA (tracrRNA) and together form a complex with Cas9 nuclease. Genomic cleavage by CRISPR-Cas9 is specific only to sequences adjacent to the protospacer motif or protospacer adjacent motif (PAM) sequence. CRISPR DNA loci are not cleaved because they are not next to a PAM sequence.

CRISPR-Cas9 has become a powerful genome editing technique with the adaption of the type II CRISPR-Cas9 systems from *Streptococcus pyogenes*. In this simplified system, the crRNA and tracrRNA are fused together into one small guiding RNA (sgRNA). For this sgRNA 20 nucleotides immediately adjacent to the 5' of the PAM sequence signify the complementary DNA binding sequence of the CRISPR-Cas9 system. While it has been reported that there are many PAM sequences that can be used for Cas9 targeting, the most effective and highly conserved sequence is 5' NGG. This has given scientist the ability to cause targeted double-stranded break anywhere in the genome provided it follows the pattern 5' N_{20}-NGG.

Materials and Methods

Mosquito Rearing

Genomic DNA was extracted from the stock colony of *Culex pipiens* form *pipiens* strain from Baylor University. Our lab strain is an off branch of the Buckeye Strain of *Cx. pipiens* mosquitoes from the Denlinger lab at The Ohio State University. The Denlinger lab started the colony in September 2000 by collecting *Cx. pipiens* larvae in Columbus, Ohio. Larval *Cx. pipiens* are raised in plastic tubs with de-chlorinated tap water and fed Tetramin fish food (Tetra holding (US) Inc., Blacksburg, VA, USA) and liver powder. Adults are maintained on Stakich Raw honey (Stakich, Inc Royal Oak, MI, USA) and kept in screened cages (55 cm X 60 cm X 65 cm). *Cx. pipiens* are kept in a room with control temperature at 25 °C, 75% relative humidity, and a 15 hour light: 9 hour dark daily light cycle.

Genomic DNA Extraction

Cx. pipiens DNA extraction was preformed using the QIAGEN DNeasy Blood and Tissue kit (Cat. # 69506).

1. 10-15 Female *Cx. pipiens* were collected into a 2 mL microcentrifuge tube
2. 180 µl of Animal Tissue Lysis buffer and Pestle sample
3. Add 20 µl of proteinase K, vortex and spin down on a low power centrifuge
4. Shake for 30 minutes at 240 rpm then incubate at 50 °C over night
5. Next day vortex for 15 seconds
6. Add 200 µl of Animal Lysis Buffer
7. Add 200 µl of 100% ethanol
8. Vortex sample

9. Transfer sample to a DNeasy mini-spin column and centrifuge at 6000 g for 1 minute
10. Remove eluted waste
11. Add 500 µl of Animal Wash buffer 1, centrifuge at 6000 g for 1 minute
12. Remove eluted waste
13. Add 500 µl of Animal Wash buffer 2, centrifuge at 16,000 g for 6 minute
14. Remove eluted waste
15. Place filter basket into a 1.5 mL tube
16. Add 100 µl of Animal Elution buffer
17. Leave at room temperature for 1 minute
18. Centrifuge at 6,000 g for 2 minute
19. Collect the elution

Concentration and quality of extraction just checked using a Nanodrop2000.

$$260/280 \approx 1.80 \; 260/230 \approx 2.0$$

Designing Primers for Ruby Amplicon

Kynurenine 3-monooxygenase Gene ID: CPIJ017147 (*ruby*) was chosen as the target for cas9 cleavage due to *ruby* knockout mutants expressing a Ruby eye [12]. This allows for us to immediately determine if the CRISPR-Cas9 system is working. We target the fourth exon of *ruby* because it is the largest of the seven exons. Primers were designed to amplify the fourth exon as well as the surrounding introns and a small sequence of the adjacent exons 3 and 5 shown in Figure 3.1.

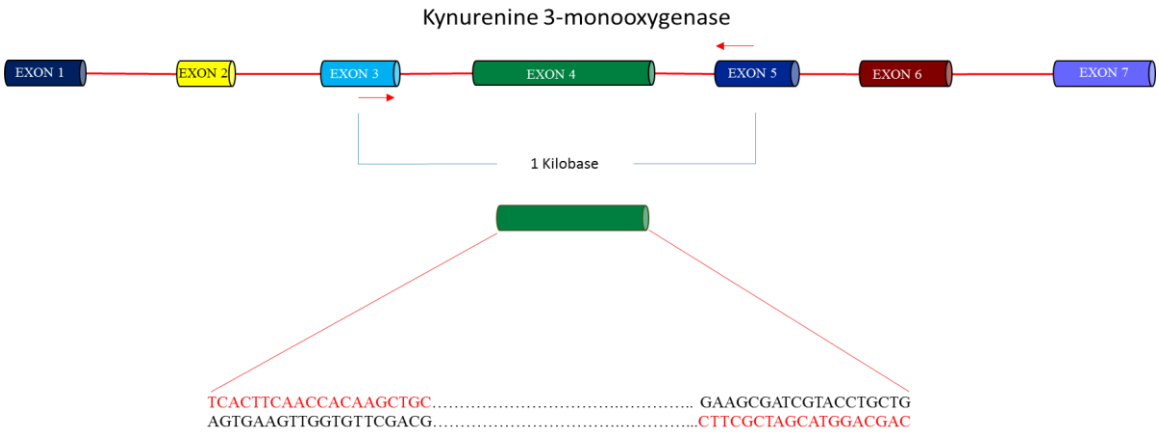

Figure 3.1: Expected amplified region of *ruby* ranging from exon 3 to exon 5 creating a 1 kb amplicon

Size confirmation of *ruby* PCR amplicon was determined using 2% agarose gel electrophoresis (Table 3.1).

Table 3.1 PCR reaction set up ruby DNA Amplicon

Reactants	Volume	Temp	Time
DH_2O	40.5 µl	95 °C	120 seconds
10x NEB Taq Buffer	5.0 µl	**45 cycles**	
100 µm DNTPs	1.0 µl	95 °C	30 seconds
Culex pipiens gDNA	1.0 µl	68 °C	90 seconds
100 µm Ruby Forward primer	1.0 µl	**1 cycle**	
100 µm Ruby Reverse Primer	1.0 µl	68 °C	120 seconds
NEB Taq Polymerase	0.5 µl	**Hold**	
Total	50.0 µl	4 °C	∞

Ruby PCR product was purified using the QIAGEN minielute PCR Purification kit (Cat. No. 28006).

1. In a QIAquick column 2 mL collection tube add Buffer PBI and PCR product together in a 5:1 ratio respectively.
2. Centrifuge for 1 minute at 16,000 g
3. Discard elute
4. Add 750 µl of PE (Wash) Buffer
5. Centrifuge for 1 minute at 16,000 g
6. Discard elute
7. Without adding anything to the column, centrifuge for 1 minute at 16,000 g
8. Discard collection tube
9. Place QIAquick collection tube into a 1.5 mL microcentrifuge tube
10. Add 50 µl of EB buffer (10 mM tris Cl, 8.5 pH) to collection tube.
11. Leave at room temperature for 1 minute
12. Centrifuge at 16,000 g for 2 minute
13. Collect the elution

Concentration and quality of purified product was checked using a Nanodrop2000.

$$260/280 \approx 1.80 \; 260/230 \approx 2.0$$

Designing CRISPR sgRNA for Targeting Kynurenine 3-monooxygenase

Unlike Zinc Finger Nucleases (ZFN) and Transcription activator-like effector nucleases (TALENs) which rely on proteins with DNA binding domains to guild nucleases to DNA cleavage sights, the CRISPR-Cas9 system uses base pairing between the CRISPR sgRNA and genomic DNA. CRISPR sgRNA can be design to target almost any sequence is the genome. CRISPR sgRNA must have a 20-16 over lapping region at the 5' prime end of the sgRNA. DNA regions being targeted must also have a 3 nucleotide protospacer

adjacent motif (PAM) sequence (NGG). It is very important to remember not to incorporate the PAM sequence into sgRNA design. It should only be on the DNA being targeted. Target sites can be determined manually be scanning the genome for PAM sites on either strand. Target sites can also be generated from multiply online tools such as ZiFit http://zifit.partners.org/zifit/choicemenu.aspx or CRISPR Design http://crispr.mit.edu. Even though both of these tools have the *Aedes aegypti* and *Anopheles gambiae* genomes preprogram, since our work is done in *Cx. pipiens* we searched for targets manually. To have to highest rate of mutagenesis we targeted two locations for Cas9 cleavage. Targeted sites were AAAACAGGCCGATCTGATCGTGG AGG and GATGCGGCACACGCCATGG GGG.

Synthesis of CRISPR sgRNA DNA Template

The template for sgRNA was generated using a modified method of the dual primer annealing method first introduced by [13]. This method anneals together two ssDNA molecules that come together to make the template for the sgRNA. The CRISPR forward primer (CRISPR F) is composed of a 24 nucleotide T7 promoter region, the 20 -16 nucleotide targeting sequence, and 20 nucleotides of the Cas9 binding domain. The CRISPR reverse primer (CRISPR R) can be used as a universal primer to anneal to all CRISPR forward primers. CRISPR R is composed of 20 nucleotide Cas9 binding region over lapping with CRISPR F followed by the rest of the Cas9 binding domain and the termination sequence.

- CRISPR R:

  5'–AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG

  GACTAGCCTTATTTTAACTT**GCTATTTCTAGCTCTAAAAC**–3'

– CRISPR F:

5’–GAAATTAATACGACTCACTATAGGAAAACAGGCCGATCTGA

TCG**GTTTTAGAGCTAGAAATAGC** –3’

Sequences highlighted in blue represent T7 RNA polymerase binding sequence, Sequences in red represent complementary DNA target sequence, sequences in black represent complementary regions between primers, normalized color represent Cas9 binding domain.

Table 3.2 PCR reaction set up CRISPR DNA Template

Reactants	Volume	Temp	Time
DH_2O	33.5 µl	95°c	2 minutes
10x NEB Taq Buffer	5.0 µl	**45 cycles**	
100 µm DNTPs	1.0 µl	95°c	20 seconds
100 µm CRISPR Forward primer	5.0 µl	60°c	10 seconds
100 µm CRISPR Reverse Primer	5.0 µl	68°c	10 seconds
NEB Taq Polymerase	0.5 µl	**Hold**	
Total	50.0 µl	4°c	∞

CRISPR template PCR product was purified using the QIAGEN mini elute PCR Purification kit (Cat. No. 28006).

1. In a QIAquick column 2 mL collection tube add Buffer PBI and PCR product together in a 5:1 ratio respectively.
2. Centrifuge for 1 minute at 16,000 g
3. Discard elute
4. Add 750 µl of PE (Wash) Buffer
5. Centrifuge for 1 minute at 16,000 g

6. Discard elute
7. Without adding anything to the column, centrifuge for 1 minute at 16,000 g
8. Discard collection tube
9. Place QIAquick collection tube into a 1.5 mL microcentrifuge tube
10. Add 50 µl of EB buffer (10 mM tris Cl, 8.5 pH) to collection tube.
11. Leave at room temperature for 1 minute
12. Centrifuge at 16,000 g for 2 minute
13. Collect the elution

Concentration and quality of purified product was checked using a Nanodrop2000.

$$260/280 \approx 1.80 \; 260/230 \approx 2.0$$

In Vitro Transcription of CRISPR sgRNAs

To generate CRISPR sgRNA we used the T7 Megascript Kit set up for in vitro transcription reaction with the following the manufacturer's protocol. Reaction ran for 4 hours at 37°c. 1 µl of DNase was added to degrade the remaining DNA (Table 3.3).

Table 3.3 Reaction set up for in vitro CRISPR sgRNA

Reactants	Volume	Concentration
DH_2O	-	
10x Reaction Buffer	2.0 µl	
RNTPs	8.0 µl	75 mM
sgRNA PCR template	8.0 µl	100 µm
T7 enzyme mix	2.0 µl	
Total	20.0 µl	

Trizol/phenol-chloro form RNA Extrantion Method

1. Add 15 µl of nuclease free water
2. 15 µl of ammonium acetate or sodium acetate
3. 800 µl of trizole
4. incubate at room temperature for 5 minutes
5. Add 200 µl of chloroform-phenol and then mix well
6. Centrifuge at 12,000 g for 10 minutes at 4c
7. Transfer the aqueous phase (RNA) to a fresh microcentrifuge tube
8. Add 500 µl of isopropyl alcohol
9. incubate at room temperature for 10 minutes
10. Centrifuge at 12,000 g for 10 minutes at 4c
11. Descant supernant
12. Add 750 µl of ethanol
13. Mix and Centrifuge at 7,000 g for 5 minutes at 4c
14. Descant supernant
15. Dry remaining ethanol
16. Resuspend RNA in nuclease free water

Cas9 Protein

We were unable to express our own Cas9 protein. Therefore we purchase recombinant Cas9 protein from PNA Bio (http://www.pnabio.com) catalog #CP01).

In Vitro CRISPR Cas9 Digest Reaction

The cas9 digest and double digest reactions requires the CRISPR sgRNA(s) synthesize previously described, Cas9, the DNA target, and a cleavage buffer. Reaction condition for the reaction is set up (Table 3.4).

Table 3.4 CRISPR-Cas9 Endonuclease digest of *ruby*

Reactants	Volume	Concentration
DH_2O	9.5 µl	
NEBuffer 3	2.0 µl	
CRISPR 1 or 2 (1.4 mM)	1.0 µl	70 ng/µl
ruby amplicon 2 (200 mM)	1.0 µl	10 ng/ µl
PNA bio Cas9 Protein	6.5 µl	32.5 ng/µl
Total	20.0 µl	

Double digest is set up similarly to the single digest with the addition of a second CRISPR sgRNA (Table 3.5).

Table 3.5 CRISPR-Cas9 endonuclease double digest of *ruby*

Reactants	Volume	Concentration
DH_2O	8.5 µl	
NEBuffer 3	2.0 µl	
CRISPR 1 (1.4 mM)	1.0 µl	70 ng/µl
CRISPR 2 (1.4 mM)	1.0 µl	70 ng/µl
ruby amplicon 2 (200 mM)	1.0 µl	10 ng/ µl
PNA bio Cas9 Protein	6.5 µl	32.5 ng/µl
Total	20.0 µl	

Set Up of Forced Laying

Blood feed females 3 days before injections. Embryos can be collected from rafts laid in the main screen, there is no need to separate embryos used for injections.

Pre-Embryo Injection

1. Use wet filter paper to remove rafts from water cups.
2. Using a piece of double sided tape align the raft horizontal onto the tape.
3. Embryos must be a lined facing the same orientation (all having their anterior and posterior ends facing the same direction
    a. Embryos must be injected into the posterior end.

Note: The anterior end of the embryo is slightly wider than the posterior.

4. Roll the raft backward and forward on the tape until only one egg thick.
5. Place tape on a microscope slide
6. Desiccate (let dry) the embryos about 1 min. at room temperature. They start to dimple slightly as they dry.
7. Add a drop of water or micro injection buffer
8. Place slide on a compound microscope.
9. Mosquitoes injected at 45 minutes after being laid have the highest survival rate [24].

Microinjection of the Embryos

1. The most important aspect of good injection is the quality of the needle. Needle must be sharp.

2. Fill a needle with the CRISPR-Cas9 solution to be injected by using a microloader. (Very little injection solution is needed: 1 to 2 µl).
3. Inject 0.2-0.5 nl of injection material into embryo.
    a. Inject embryos horizontally or on an angle.
    b. Penetration should be at the posterior end of mosquito embryo.
4. Pick embryos from the slide using fine forceps or a fine brush, and place them in a plastic water cup and return to insectary conditions for 4 days.

Post-injection

1. After four days place embryo check for any embryos that hatched.
    a. Be sure to add a small amount of food
    b. Embryos will continue to hatch over a very long period so don't throw out the culture until at least 4-5 weeks after injection.

Results

Preliminary genomic screening of target region can be done by extracting gDNA from *Cx. pipiens* and amplification by PCR. *Cx. pipiens ruby* was chosen for cleavage by CRISPR/Cas9. Knocking out *ruby* in *Cx. pipiens* create yellow body larvae and ruby eyed adults [22]. This mutant phenotype can be easily detected by visual observation. We amplified a 1 kb region of the *ruby* gene by PCR followed by purification Figure 3.2.

The CRISPR/Cas9 ratio can be optimized in vitro prior to insertion in vivo. An advantage of in vitro optimization is the decrease in cost and time. Synthesis of the CRISPR sgRNA was achieved by annealing two single stranded oligo DNA strands together. The sequence of strand 1 contained a T7 RNA polymerase promoter, complementary DNA

binding sequence of our target, and 20 nucleotide overlap region with strand 2. Strand 2 contained the complementary 20 nucleotide sequence and the in Figure 3.3.

A

ruby Forward Primer #1
-TCACTTCAACCACAAGCTGC
ruby Reverse Primer
-CAGCAGGTACGATCGCTTC

B

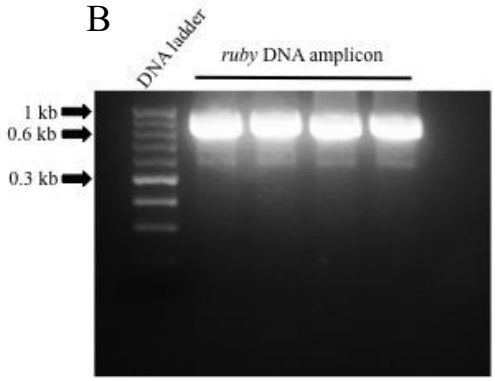

Figure 3.2: Size confirmation of *ruby* DNA A) Primer sequences of forward primer and reverse primer used to amplify *ruby* (CPIJ017147). Product of amplification equal 995 bp. B) Agarose gel electrophoresis for *ruby* PCR amplification. Lanes 2-5 show the bands of the *ruby* gene (~ 1kb) which were amplified directly from gDNA extracted from *Cx. pipiens*.

Cas9 endonuclease becomes catalytically activated at temperatures 30-37 °c [28]. In vitro digest requires only the sgRNA, Cas9 protein, and DNA cleavage target. Our in vitro protocol is specific to low volumes thus reducing the cost of each reaction while still reporting DNA cleavage. Figure 3.4A reports single Cas9 DNA digest with sgRNA 1 and sgRNA 2. It was unexpected to find a band suggestive of a DNA fragment below 200 bp. We hypothesize that it may be the result of sgRNA bound to Cas9. To test this we incubated sgRNA and Cas9 at 25 °c. Since Cas9 does not become catalytically active until temperatures between 30 °c and 37 °c cleavage will not occur.

Cas9 is shown to bind sgRNA at 25 °c to test if the band appearing around 200 bp on a gel was CRISPR bound to Cas9 we ran the digest reaction at 25 °c figure 3.4B. Cas9 has the ability to bind multple sgRNAs. To test this in vitro we introduce both sgRNA

A

GAAATTAATACGACTCACTATAGGAAAACAGGCCGATCTGATCGGTTTTAGAGCTAGAAA
TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC
TTTT
CRISPR #1 Sequence: Target Sequence
GAAATTAATACGACTCACTATAGGCGGGGATGCGGCACACGCCAGTTTTAGAGCTAGAAA
TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC
TTTT
CRISPR #2 Sequence: Target Sequence

B

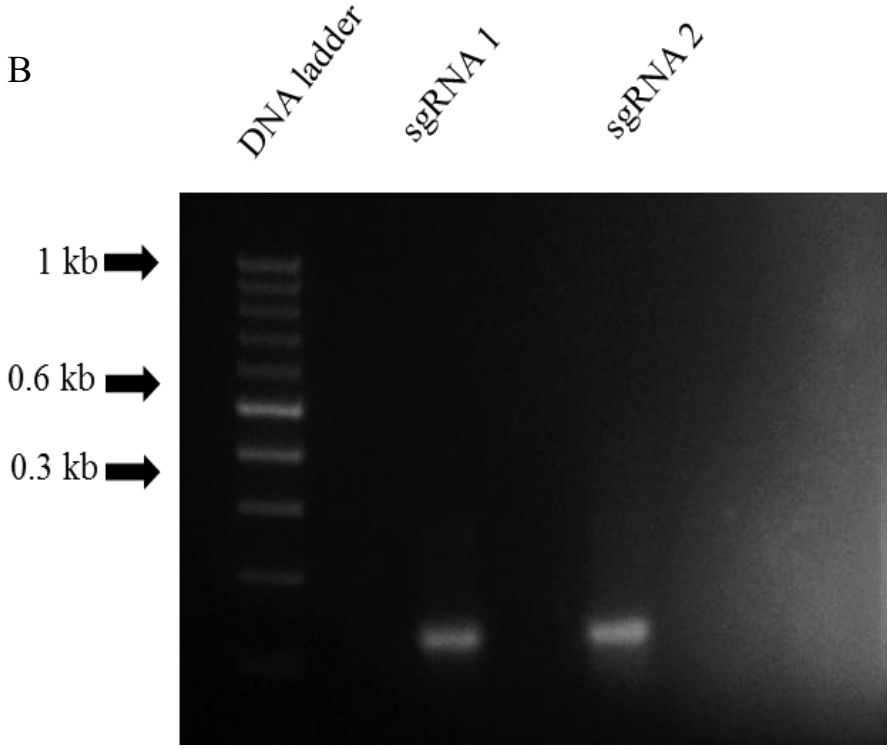

Figure 3.3. Size confirmation of sgRNA DNA template. A) DNA sequence of guide RNAs. B) CRISPR template PCR product purification. Expected band size is 124 bp for both guiding RNAs.

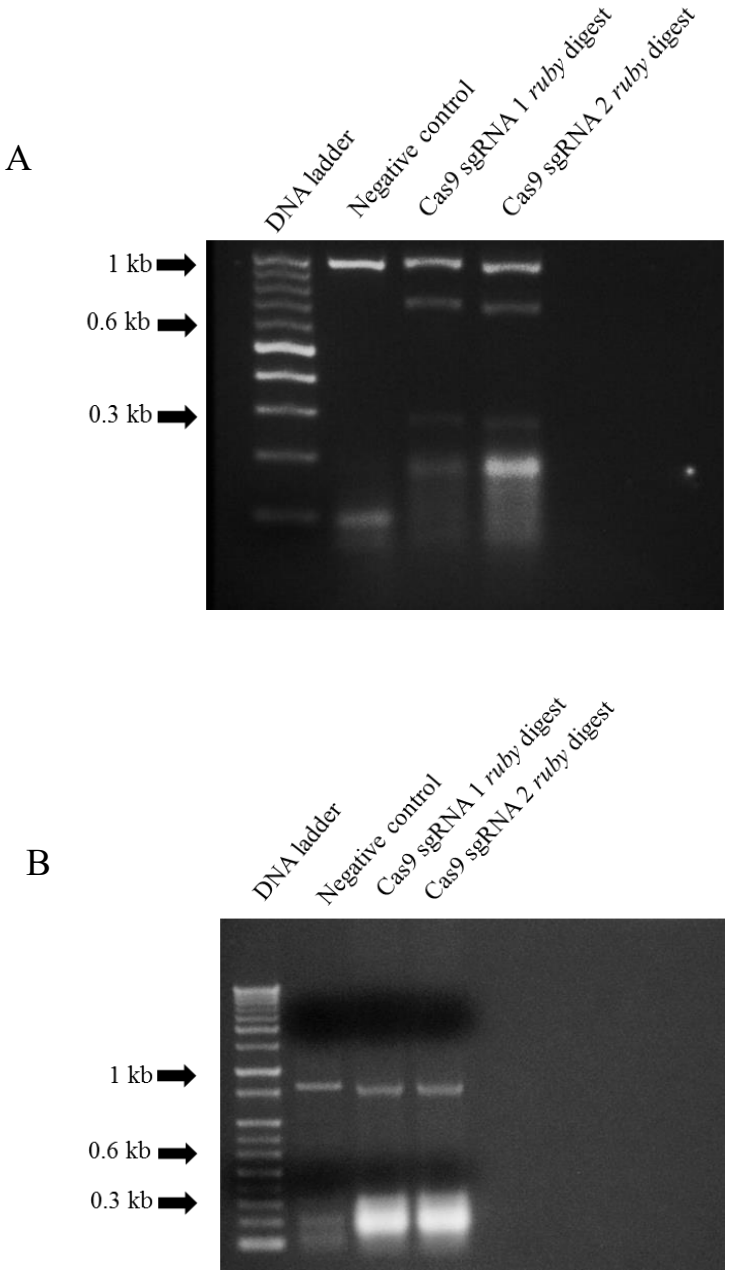

Figure 3.4. CRISPR/Cas9 digest of *ruby* using a single sgRNA A) CRISPR/Cas9 digest ran at 35 C B) CRISPR/Cas9 digest ran at 25 C to determine sgRNA binding without DNA cleavage.

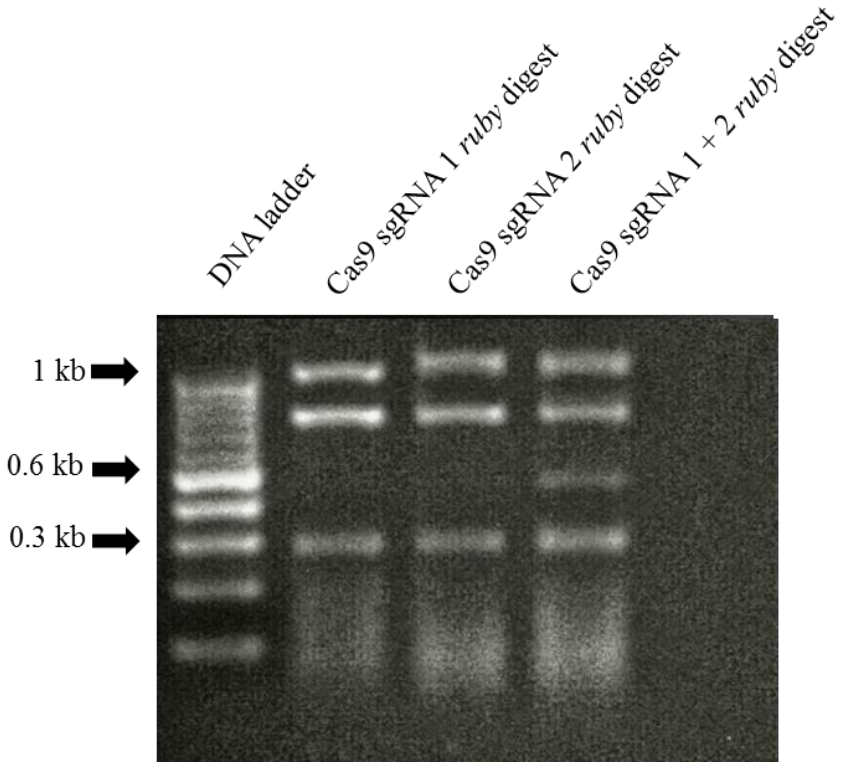

Figure 3.5. CRISPR/Cas9 double digest of *ruby* using two guide RNAs. Cas9 guide cleavage of ruby gene was done using a single sgRNA, Lane 2 and Lane 3 and by two sgRNA in Lane 4. Expected fragment sizes of double digest are 440 bp and 280 bp. 1 kb band and 700 bp band show undigested DNA and undigested single cleaved DNA respectively.

to one reaction. Band of 1 kb and 700 bp represent incomplete double digest Figure 3.5.

Summary

CRISPR-Cas9 technology has been undergoing a series of rapid change. Early adaptions of CRISPR RNA gave rise to sgRNA, a fusion of the crRNA DNA binding domain and the tracrRNA Cas9 binding domain [25]. More recent adaptations of the CRISPR-Cas9 system involve modifications to Cas9 protein. Inactivation of the RuvC1 domain or HNH nuclease domain [26, 27]. RuvC1 inactivation results in complementary strand cleavage and HNH inactivation results in noncomplementary strand cleavage [26,

27]. Double inactivation of RuvC1 and HNH results in a completely inactive Cas9 protein capable of recognizing DNA strands however no cleavage will be achieved [25,28].

The use of an in vitro CRISPR/Cas9 digest to determine cleavage may eventually replace indel sequencing. We use a simplified method to synthesize the CRISPR/Cas9 system. Doing so has given us the ability develop an in vitro CRISPR/Cas9 digest. Our primary purpose of in vitro digest using CRISPR/Cas9 is to determine the functionality of our synthesized system prior to in vivo experimentation. A weakness of this method is that it can only be used alongside an in vitro transcription CRISPR and Cas9 in vitro translation techniques, in other words it is necessary to have the active Cas9 and the guide RNA. Methods using a plasmid expression vector of Cas9 or CRISPR to preform genomic editing will need to express the necessary components prior to the in vitro digest method.

CRISPR Cas9 technology has been established in *Aedes* mosquitoes [23]. However, the *Culex* mosquito complex still lacks the editing power of the CRISPR/Cas9 system. In the future we plan to establish a genetic knockout system in *Culex* germ-line cells. Our methods will to the direct injection of CRISPR/Cas9 into the mosquito embryo. Targeted genomic editing during the embryo stage will carrier all mutations made into all somatic and germ cells. This mutation can then be passed down in germline onto its offspring. Mosquitoes produce high numbers of progeny after mating thus increasing the amount of knockout mutants we can observe.

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CHAPTER FOUR

Conclusions

Cx. pipiens are the major disease vector affecting many regions around the world. Millions of people are at risk for diseases caused by *Cx pipiens* every year. This increases the need for an effective method to study *Culex* and diseases they carry. In chapter 2 we study lymphatic filariasis in *Cx. pipiens* and found candidates genes to be studied as a transmission-blocking vaccine. We used RNA deep sequencing and qRT-PCR to measure and validate differences in the transcriptome of *B. pahangi*. To further determine the ability of these genes for a transmission blocking vaccine candidates they will be expressed in two eukaryotic expression systems, the yeast expression system and the baculovirus/insect cell system. The expressed and purified recombinant proteins will be characterized for identity, conformation (folding), yield, purity, and functional activity. These high-quality recombinant proteins will then be used for pre-clinical trial using *B. pahangi* and *Cx. pipiens* for the future proposed studies.

In chapter three we develop an in vitro CRISPR Cas9 system to determine activity of synthesized CRISPR/Cas9 systems. This preliminary screening systems works to supplement the work that will be done in vitro and will require more development before it can stand on its own. The future work to chapter three will involve developing the CRISPR/Cas9 system in *Cx. pipiens* to establish a germ-line knockouts system. Our methods will be to the directly inject CRISPR/Cas9 into the mosquito embryo and edit the genome of *Cx. pipiens*.

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