Determination of a Gene Expressed only in the Infective Larval Stage of *Dirofilaria immitis*

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ABSTRACT

Current molecular assays for detecting *Dirofilaria immitis* DNA in mosquitoes by PCR cannot differentiate between infected mosquitoes that contain any stage of the parasite and infective mosquitoes that harbor third-stage parasite larvae (L3). This over-estimates transmission risks to canines because only L3 *D. immitis* are capable of establishing infections in canines. I have developed an assay that detects *D. immitis* L3 in the vector *Aedes aegypti* based on a reverse transcriptase-polymerase chain reaction (RT-PCR) method that detects an L3-activated gene transcript. Potential L3 stage genes were identified using bioinformatics tools and were screened by PCR using complementary DNA (cDNA) libraries as templates. Candidate genes were screened for stage-specific expression using RNA isolated from both *D. immitis* infected and uninfected mosquitoes. RT-PCR was performed using primer sets specific for each candidate gene. The L3 specific gene transcript CK855471A was only detected in infective mosquitoes. This L3-activated gene transcript and a constitutively expressed control transcript, *Ditph*, for all vector-stage filarial larvae can be used to detect filarial infectivity in pools of 25 mosquitoes. This assay may be useful for examining the seasonality of transmission, and *D. immitis* gene regulation.

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Most of all, I appreciate my immediate family to whom this thesis is dedicated. They have been a constant source of love, concern, support and strength throughout the years.

DEDICATION

This thesis is dedicated to my loving parents, Melody Satchell and Simon Thompson, who have raised me to be the person I am today. They have been a source of motivation, strength and support during moments of despair and discouragement. I know I have made you proud and this thesis is only the beginning of my journey.

"We all have dreams. But in order to make dreams come into reality, it takes an awful lot of determination, dedication, self-discipline, and effort." – Jesse Owens

Chapter I

INTRODUCTION

Dog heartworm is a canine disease caused by infection with the filarial nematode *Dirofilaria immitis* (Ledesma and Harrington 2011). This mosquito vector-transmitted parasite commonly infects dogs, but infections also have been detected in cats (Bowman and Atkins 2009), coyotes (Sacks et al., 2004), foxes, sea lions, penguins (Sano et al., 2005) and even humans under very rare conditions (Simón et al., 2009; Ledesma and Harrington 2011). Sixty-three species of mosquitoes are thought to be potential vectors of *D. immitis*, including twenty-eight species in North America (Ludlam et al., 1970). Although once confined to the southern United States, *D. immitis* now occurs in most locations where potential vectors are found (Acha and Szyfres 2003; Licitra et al., 2010). The majority of the vectors belong to the genera *Aedes*, *Anopheles* and *Culex* (Ludlam et al., 1970). Although over sixty mosquito species have been identified as vectors, the vector competence of most species remains unknown.

The risk of canine infection by *D. immitis* depends on a combination of several factors related to the definitive and intermediate hosts (Capelli et al., 2013). The likelihood of infection in the intermediate host depends on vector competence, host seeking activity, feeding preference and vector densities (Capelli et al., 2013). Heartworm infections pose a significant risk to canines because any infection is sufficient to produce pulmonary vascular and parenchymal disease (Brown et al., 2012). Chronic

infections are caused by *D. immitis* adults residing within the right ventricle and pulmonary arteries (Licitra et al., 2010). Many dogs show little or no sign of infection even after the worms become adults although one early sign is a cough. In more advanced cases, where worms may have accumulated in the heart without treatment, more severe symptoms occur such as weight loss, fainting, and congestive heart failure (Bowman and Atkins 2009). The Companion Animal Parasite Council (CAPC) examined results of 4,769,403 canine heartworm tests during the 2011 calendar year and found that 56,612 (1.2%) had heartworm antigens indicating an active *D. immitis* infection (Brown et al., 2012). Despite improved diagnostic methods, increased awareness among veterinarians and pet owners, and effective preventives, heartworm infections are diagnosed frequently and are becoming more prevalent in areas previously considered to be at low risk (Bowman et al., 2009). In order to reduce *D. immitis* incidents researchers must better understand its lifecycle.

The complete lifecycle of *D. immitis* consists of four developmental stages: microfilariae (Mf), larvae (L1-L4), juvenile adults and adult worms. Dogs, the definitive host, become infected when third stage larvae (L3) exit the labium of infected mosquitoes, the intermediate host, during a blood meal. The L3 move across the host's skin and enter through the mosquito feeding site. At the site of inoculation the L3 molt to fourth stage larvae (L4) in the muscles and submuscular membrane of the definitive host. The larvae develop into juvenile adults, enter the bloodstream through the surrounding vasculature and migrate to the host's pulmonary arteries. Adult worms reproduce sexually, and microfilariae produced by gravid females are released into the peripheral blood (Ledesma and Harrington 2011).

Mosquitoes become infected with *D. immitis* when they ingest the Mf while blood feeding on an infected dog (Brown et al., 2012). After ingestion, Mf migrate from the mosquito's midgut to the Malpighian tubules in the abdomen. There the parasites develop into the first-stage larvae (L1) and second-stage larvae (L2) (Ledesma and Harrington 2011). The L2 exit the Malpighian tubules and migrate through the mosquito body cavity (hemocoel) from the abdomen to the head where they molt to the infective (L3) stage. The L3 move to the mosquito's proboscis, emerge from the labium when it takes a blood meal and infects another definitive host (Ledesma and Harrington 2011). To be a competent vector of *D. immitis*, mosquitoes must support nematode development through the L3 stage. Not all Mf ingested by mosquitoes survive and become infective L3 (Laney et al., 2010).

In the past, potential vectors were identified by dissecting field-collected mosquitoes to look for L3 development (Licitra et al., 2010). This technique is labor intensive, not conductive to identifying life stages, and impractical for screening large numbers of mosquitoes (Sauerman and Nayar 1983). An alternative diagnostic test for *D. immitis* DNA using polymerase chain reaction (PCR) was developed to identify infective mosquitoes (Scoles and Kambhampati 1995; Watts et al., 2001). Studies by Chambers et al. (2009) and Fisher et al. (2007) that compared these two techniques found that PCR produced a higher detection rate than dissection and allowed more samples to be analyzed simultaneously while reducing human error. Unfortunately, the Scoles and Kambhampati (1995) assay detects DNA from all parasite stages and therefore does not accurately measure population transmission potential because it does not differentiate between infected and infective mosquitoes. Thus, the Scoles and Kambhampati (1995)

PCR estimates parasite prevalence rather than infectivity because the Mf ingested by a mosquito do not all survive and develop into infective L3 (Laney et al., 2010). To directly assess transmission potential the presence of L3 in a vector population must be evaluated.

An assay that specifically targets *D. immitis* L3 would be useful for answering research questions about the seasonality of transmission and *D. immitis* gene regulation. Such an assay would also be valuable for identifying or confirming mosquito species that are vectors in geographic regions where heartworm is emerging. Recent studies have found new geographic regions in Europe (e.g., Italy Spain and Turkey) where previously unknown vectors (e.g., *Culex pipiens*) of *D. immitis* have been identified (Capelli et al., 2013; Morchón et al., 2007; Yildirim et al., 2011). The discovery of new vector species provides further rationale for the development of a highly sensitive method for monitoring transmission potential. The research reported here identifies a *D. immitis* L3 stage-specific activated gene and an L3 detection assay for the presence or absence of infective stage *D. immitis* in mosquito vectors using reverse transcriptase polymerase chain reaction (RT-PCR).

Chapter II

MATERIALS AND METHODS

Search Strategy for L3-activated Candidate Genes

Cuticlin genes in nematode parasites that are activated at the L3 stage (Liu et al., 1995) have been successfully used as markers for identifying L3 stage parasites in the human filarial worms *Brugia malayi* (Laney et al., 2008) and *Wuchereria bancrofti* (Laney et al., 2010). Therefore, selection of potentially diagnostic *D. immitis* candidate genes focused primarily on identifying *D. immitis* genes orthologous to *B. malayi* and *W. bancrofti* cuticle genes. Supplementary L3-activated gene sequences from *B. malayi*, *W. bancrofti*, and *Caenorhabditis elegans*[1] databases were also screened. Potential candidate gene sequences were identified by bioinformatics analysis of Expressed Sequence Tagged (EST) databases using nucleotide Basic Local Alignment Search Tool (BLASTN) available at http://xyala.cap.ed.ac.uk/downloads/959nematodegenomes/blast/ /filareu.php and screened against the *D. immitis* nuclear genome assembly 2.2 DNA sequence (Godel et al., 2012). Although, identification of L3-activated gene sequences was the primary objective, genes also expressed in the L4 through adult stages were not excluded.

Primer Design

[1] *Caenorhabditis elegans* dauer stage is thought to be analogous to the L3 stage of parasitic nematodes.

Potential target genes were screened using the mRNA-to-genomic DNA alignment program, SPIDEY (www.ncbi.nlm.nih.gov/spidey) (Wheelan et al., 2001),

which readily identifies intron-exon junctions in order to prevent detection of genomic DNA (gDNA) in complementary DNA (cDNA) library preparations (Laney et al., 2008; Laney et al., 2010). Specific primers were designed for each candidate gene using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012). The criterion used to create each primer was as follows: 1) one primer for each gene spanned an exon-exon boundary to prevent the amplification of gDNA, 2) sequence length ranged between 17 and 23 nucleotides, 3) melting temperatures (T_m) were between 55 °C and 60 °C, and within 2 °C of each other, 4) GC content ranged between 33.0% to 69.0%, 5) When possible, G or C bases were within the last 5 bases from the 3' end to promote GC clamp binding, 6) product sizes ranged between 190 and 570 base pairs (bp), and 7) when possible, self-complementarity values below 8 and self 3'complementarity values lower than 6 were used to avoid primer dimers and hairpins. At least one primer set was created for each candidate gene. More primer sets were created depending upon the number of intron-exon boundaries detected. All primer sequences used in this study are listed in Table 1.

Pre-Screening of Candidate L3-activated Genes using mRNA Libraries

Converting mRNA libraries to cDNA libraries was performed using the SuperScript® IV First-Strand Synthesis System Kit (Invitrogen Life Technologies, Grand Island, NY) following the manufacturer's protocol. Each reaction (20 µL) included 50 µM Oligo $d(T)_{20}$ primer, 10mM dNTP mix, DEPC-treated water, 5x SSIV Buffer, 100 mM DTT, RNaseOUT™ Recombinant RNase Inhibitor and SuperScript® IV Reverse Transcriptase. PCR used template (5 µL) from stage-specific mRNA libraries: DiMf and DiL3 (available at http://www.filariasiscenter.org/). Candidate genes were screened for

L3 stage-specific expression by RT-PCR with the Platinum® *Taq* DNA Polymerase kit (Invitrogen Life Technologies, Grand Island, NY). The Platinum® *Taq* DNA Polymerase kit was used according to manufacturer's instructions except that a 2.5 mM dNTP mix was used instead of 10 mM (volume used was equivalent to 10 mM), and the total volume was adjusted from 50 µL to 25 µL.

Each 25 µL PCR reaction contained buffer without magnesium chloride ($MgCl_2$), 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP (Fisher Scientific, Pittsburgh, PA, USA), 0.2 µM of the forward and reverse primers, 0.2 µM Platinum® *Taq* DNA polymerase, and 2 µL template cDNA. The PCR cycling parameters used were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension held indefinitely at 4 °C. PCR products were size fractionated by electrophoresis on 3% agarose gels (Fisher Scientific, Pittsburgh, PA) stained with GelRed (Biotium, Hayward, CA), run at 70V for 1.5 h. Results were visualized using an ImageQuant LAS 4000 (GE Healthcare, Pittsburgh, PA, USA) with ultraviolet light. The primers used in the multiplex RT-PCR for the control gene *Ditph* amplify a 152 bp fragment from all stages of the parasite (*Ditph* F 5'-AAGGTCGACAAGTGGTGAGAA-3' and *Ditph* R 5'-GTAGCCATATCTTCGTAACAGTTC-3') to ensure RNA sample degradation had not occurred. The *Ditph* primer sequence was designed based upon a *D. immitis* orthologue that was identified following bioinformatics screening of a constitutively expressed tph-1 from *W. bancrofti* (Accession # CD374712.1). Candidate genes were excluded from further consideration if DiMf amplification products were detected or if no product was detected for DiL3 cDNA library.

Parasite Infection of Mosquitoes

Aedes aegypti black-eyed mosquitoes infected with the Missouri 2005 strain of *D. immitis* was provided by the NIH/NIAID Filariasis Research Reagent Resource (FR3) Center at the University of Georgia (http://www.filariasiscenter.org). Animals used by the FR3 Center (www.filariasiscenter.org) were handled according to the NIH guidelines for animal experimentation and the animal experimental protocols were approved by the University of Georgia and the University Institutional Animal Care and Use Committee under the protocol number: A2013 11-009, respectively. The animal care and use protocols adhered to the "Guide for the Care and Use of Laboratory Animals (the Guide)" published by the National Research Council, USA. The mosquitoes were fed on blood maintained at approximately 37 °C from an infected animal containing the Mf stage of the parasite using a membrane feeding apparatus. The microfilaremia in the blood ranged from 70 to 90 Mf per 20 µL. Mosquitoes were collected at 2, 5, 10 and 14 days post infection (*d.p.i*) and immediately snap frozen. These time points extend across the lifecycle of *D. immitis* and were used to examine expression profiles. The FR3 center also provided uninfected mosquitoes (unfed or after feeding on blood from uninfected cats) to serve as a control in this experiment. This study was approved by the Valdosta State University Biosafety Committee (see Appendix A and B).

RT-PCR Testing of Putative Target Genes against Mosquito Pools

RNA from pools of 25 *Ae. aegypti* mosquitoes was extracted using a total RNA isolation protocol modified (i.e., based on the Invitrogen protocol for total RNA isolation) for mosquitoes from the University of Notre Dame. See Appendix C for detailed protocol. After extraction, RNA samples (1.5 µL) were evaluated using a

NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) for purity (260/280 nanometer wavelengths) and quantity (nucleic acid concentration). RNA samples were then immediately converted to cDNA and RT-PCR was performed as described above. Potential target genes were screened against a negative RNA extraction control from a pool of uninfected mosquitoes, a positive RNA extraction control from a pool of infective 14 *d.p.i* mosquitoes, and genomic DNA (gDNA) to ensure that only the expressed *D. immitis* target genes were detected. Candidate genes were not evaluated for further testing if amplification of uninfected pools or gDNA occurred.

Time-Course Screening with L3 Specific Primers

RNA extraction, cDNA synthesis, and conventional RT-PCR, as previously described, was performed on cohorts of 25 female mosquitoes fed on *D. immitis* infected blood and held at 2, 5, 10, and 14 *d.p.i* as previously stated. Three biological repeats of all the time points were tested.

Chapter III

RESULTS

cDNA Library Multiplex RT-PCR

Table 2 lists the candidate L3-activated gene targets generated from bioinformatics searches that met the previously described criteria. Table 2 also shows stage expression for each candidate gene based on PCR amplification from a *D. immitis* Mf and *D. immitis* L3 mRNA library (*D. immitis* mRNA libraries for the L1, L2, and L4 stages were unavailable) that had been converted to a cDNA library. Five potential target genes that exhibited L3 stage expression were identified. Two genes had multiple primers created based upon different regions of the gene; CB338725 has 2 different primers (A-B) and CK855471 has 6 (A-F). Therefore, we screened eleven primer sets in total.

One of eleven target gene candidates tested (CB338725A) had transcripts expressed in the *D. immitis* Mf cDNA library and was not considered for further analysis. Four candidate genes (CK855471B, CK855471D, CK855471E and CK854857) showed no amplification in the *D. immitis* L3 cDNA library. These candidate genes were deemed to not accurately identify infective mosquitoes and were discarded. In two candidate genes, H98312 and CB338725B, PCR amplification was inconclusive by multiplex PCR due to a small difference in product sizes. These genes were tested further by conventional PCR to ensure Mf and L3 cDNA amplification had not occurred. H98312 did not show amplification for either of the *D. immitis* cDNA libraries and was

eliminated from further testing. Five target candidate genes (CB338725B, CK850096, CK855471A, CK855471C, and CK855471F) were selected for further testing as diagnostic targets based on amplification in the L3 stage cDNA library and constitutively expressed gene *(Ditph*) with no amplification in the microfilariae stage cDNA library.

Conventional RT-PCR of Candidate Genes against Mosquito Pools

The five selected gene candidates were tested using RNA isolated from infective mosquito pools collected 14 *d.p.i*, uninfected mosquito pools and gDNA from the infective mosquito pools. The *W. bancrofti* L3-activated collagen gene, CK855471A, was amplified in *D. immitis* infective mosquitoes at 14 *d.p.i* but did not amplify in genomic DNA from infected mosquitoes nor did it amplify cDNA from uninfected mosquitoes. The remaining four candidate genes were eliminated as diagnostic targets because conventional RT-PCR results indicated that none of them showed expression for infective, uninfected or gDNA mosquito pools (Table 3). The CK855471A target was determined to be the best target for an L3 detection assay.

Conventional RT-PCR of CK855471A and Ditph using Time-Course Study

High quality RNA was extracted from pools of infective mosquitoes fed on microfilaremic blood as well as uninfected mosquitoes and snap frozen using the protocol previously mentioned. The nucleic acid concentration of RNA yielded from the uninfected, 2, 5, 10, and 14 *d.p.i* time-course samples had a mean of 2718.9, 3638.3, 908.3, 2378.3, and 1986.3 ng/µL, respectively, and a standard deviation of 1000.6 ng/µL. The ratio of absorbance readings between 260 and 280 nanometer wavelengths had a mean of 2.18, 2.14, 2.16, 2.18, and 2.14, respectively, and a standard deviation of 0.02 indicating high purity RNA was extracted from all samples (Table 4).

The CK855471A primer set amplified a 286bp product from *D. immitis* Missouri 2005 stage L3, while the primer set for *Ditph* amplified a 152bp fragment from all stages of the parasite. The RT-PCR assay primers were designed to span identifiable intron-exon boundaries of *D. immitis* stage-specific candidate genes. The size difference between CK855471A and *Ditph* amplification products allowed them to be differentiated on a 3% agarose gel. Figure 1A upper panel shows expression of *Ditph* and Figure 1A lower panel shows expression of CK855471A in uninfected mosquitoes and mosquito pools at the different time points listed previously (2, 5, 10, and 14 *d.p.i*) using conventional RT-PCR. Figure 1B illustrates expression of *Ditph* and CK855471A against the same mosquito pools using multiplex RT-PCR. *Ditph* was detected in all mosquito pools, whereas CK855471A was not detected until 14 *d.p.i* in two of the three biological repeats; 10 *d.p.i* expression was found in one biological replicate. Microfilarial development to L3 can occur as early as 8 *d.p.i* (Ledesma and Harrington 2011) therefore, it is not unlikely to see L3 expression at 10 *d.p.i*; each pool of 25 mosquitoes parasite development is not synchronized. The CK855471A RT-PCR primers were specific for *D. immitis* samples; no expression was detected in mosquitoes with *D. immitis* Mf, uninfected control mosquitoes, and gDNA.

Table 1. Candidate Gene Primer Sequences.

Gene Identifier	Direction	Sequence 5'→3'
H98312	F	GAAGCACGAGAAAAAGCTTATAC
H98312	R	TCAGATGGTGGACGGTG
CB338725A	F	GTTCGAGAAACAATTCGATGGTC
CB338725A	R	CGGCTGCATCAACCTCTTTC
CB338725B	F	CTATGTCAGTTATCGATCTCGCG
CB338725B	R	CGGCTGCATCAACCTCTTTC
CK8500976	F	GCGATGATAATCAAGCTTTGCC
CK8500976	R	TTGGACGAAATTTAAACGAAATGG
CK855471A	F	GAGAACGCGCATACAAGGC
CK855471A	R	GGTTTGCAACAACTATCACAGGC
CK855471B	F	GGAGCCTGTGATAGTTGTTGC
CK855471B	R	CTGGTTCGCCCGGTTG
CK855471C	F	GGAAACAGCGGTAGTGCTG
CK855471C	R	CTGGTGGTCCATTTGGTCC
CK855471D	F	GAGAACGCGCATACAAGGC
CK855471D	R	CAGCACTACCGCTGTTTCC
CK855471E	F	CTTCAATTTTGCAAATCCTCAGC
CK855471E	R	CGCTGTTTCCTGGTCTGC
CK855471F	F	GGAGCCTGTGATAGTTGTTGC
CK855471F	R	CTGGTGGTCCATTTGGTCC
CK854857	F	CAGTGAATGTCCACGACCAC
CK854857	R	CTGGGTGACCATGATCGATG

Gene Identifier = Genebank Accession number available at http://www.ncbi.nlm.nih.gov/.
F = Forward Primer, R = Reverse Primer

Table 2. cDNA Library PCR Screening of Candidate Genes.

Gene Identifier	Putative Identification based on Protein similarity matches	DiMf cDNA Library	DiL3 cDNA Library
H98312	BM Collagen	-	-
CB338725A	BM Pyruvate	+	+
CB338725B	BM Pyruvate	-	+
CK850096	WB Cuticlin 1.0	-	+
CK855471A	WB Collagen	-	+
CK855471B	WB Collagen	-	-
CK855471C	WB Collagen	-	+
CK855471D	WB Collagen	-	-
CK855471E	WB Collagen	-	-
CK855471F	WB Collagen	-	+
CK854857	WB Cuticlin 2.0	-	-

Gene Identifier = Genebank Accession number available at http://www.ncbi.nlm.nih.gov/.
+ = PCR product detected, - = no PCR product detected
D. immitis cDNA library abbreviations: DiMf = microfilarial stage, DiL3 = infective L3 stage

Table 3. RT-PCR of Candidate Genes against Mosquito Pools.

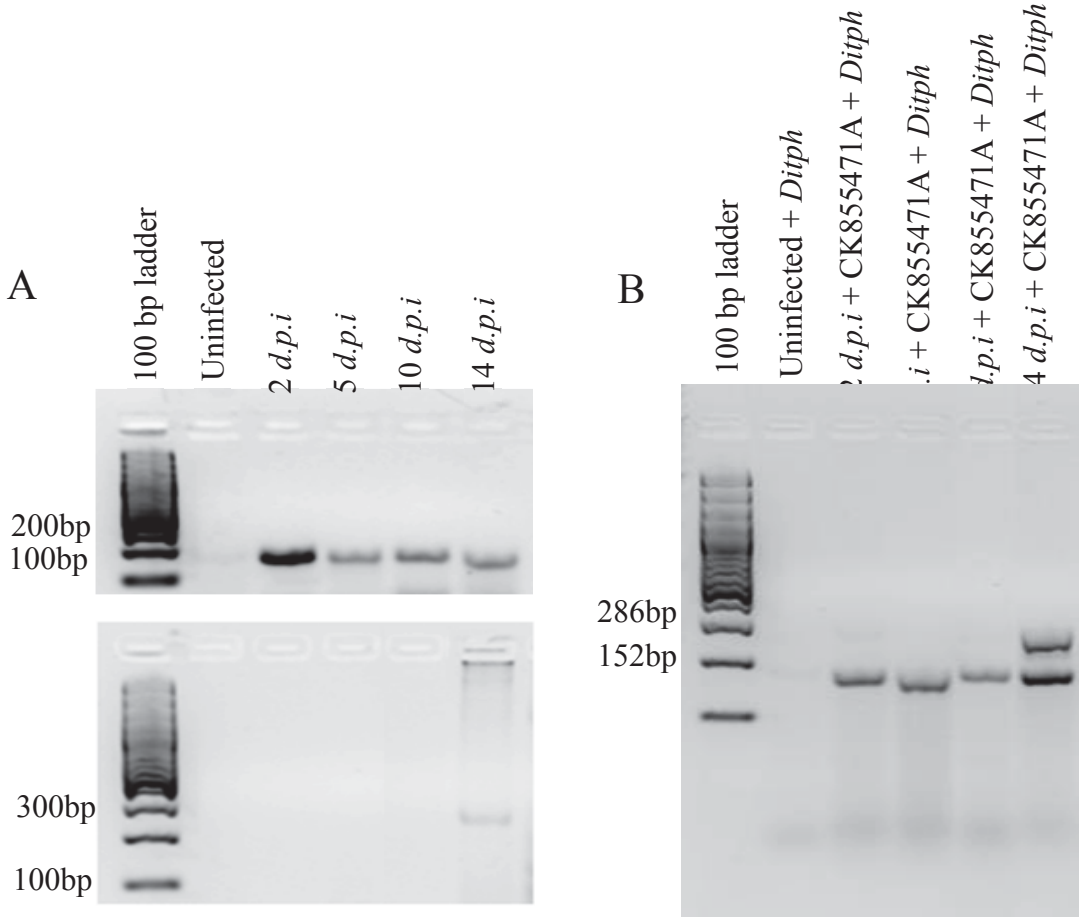
Gene Identifier	Infective mosquito cDNA	Uninfected mosquito cDNA	Infective gDNA
CB338725B	-	-	-
CK850096	-	-	-
CK855471A	+	-	-
CK855471C	-	-	-
CK855471F	-	-	-

Gene Identifier = Genebank Accession number available at http://www.ncbi.nlm.nih.gov/.
+ = PCR product detected, - = no PCR product detected

Table 4. RNA Extraction Quantity and Quality across Time-Course Study.

		ng/µL of total RNA				260/280			
Mosquito Time point (#*d.p.i*)	Expected Stage of Parasite Development	Biological Replicates				Biological Replicates			
		I	II	III	Mean	I	II	III	Mean
UI	—	3133.3	3671.6	1351.8	2718.9	2.20	2.18	2.17	2.18
2	Mf	2332.2	2564.5	6018.3	3638.3	2.15	2.07	2.20	2.14
5	L1	991.1	1235.0	498.9	908.3	2.18	2.16	2.13	2.16
10	L2/L3	2638.2	1076.5	3420.1	2378.3	2.15	2.18	2.21	2.18
14	L3	2566.1	2416.4	976.4	1986.3	2.12	2.16	2.13	2.14
				Stdev	1000.6			Stdev	0.02

# *d.p.i* = number of days post infection (after mosquitoes were feed on infected blood)
UI = Uninfected *Ae. aegypti* mosquito pool, — = no parasitic developmental stage
260/280 = RNA purity as detected by spectrophotometry
Stdev = standard deviation



200bp

Figure 1. RT-PCR Detection of *Ditph* and CK855471A in a Mosquito Time-Course.

Time course A (conventional RT-PCR) and B (multiplex RT-PCR) illustrates no amplification of the *W. bancrofti* L3-activated collagen gene CK855471A (286 bp) in pre-L3 stages, while the control *Ditph* (152bp) is detected at all time points indicating the presence of parasite RNA. Upper Panel A shows *Ditph* tested against each time point as well as a negative control (uninfected mosquitoes). Lower Panel B shows CK855471A tested against each time point as well as a negative control (uninfected mosquitoes). *d.p.i* = days post infection (after mosquitoes were feed on infected blood)

Chapter IV

DISCUSSION

In this study, I have successfully identified a *D. immitis* L3-stage specific gene that can be used for the detection of mosquitoes capable of transmitting infective stage parasites. Our search strategy for identifying an L3 stage-specific gene transcript used bioinformatics analysis of EST datasets with a primary focus on *D. immitis* orthologues to cuticlin genes from both *B. malayi* (Laney et al., 2008) and *W. bancrofti* (Laney et al., 2010). However, additional gene types (e.g. collagen and pyruvate) were also considered. This study identified five potential target genes, two of which had multiple primers created based upon different regions of the gene: CB338725 had 2 different primers (A-B) and CK855471 had six (A-F). I screened a total of eleven primer sets and examined their expression against the following: 1) *D. immitis* Mf and L3 stage cDNA libraries using multiplex RT-PCR, 2) gDNA to ensure that only the expressed *D. immitis* target genes were detected, and 3) RNA extracted from uninfected mosquitoes and infected mosquitoes (2, 5, 10, and 14 *d.p.i*) using conventional RT-PCR. The *W. bancrofti* L3-activated collagen gene CK855471A exhibited expression by conventional RT-PCR at 10 *d.p.i* in one of three biological repeats. This corresponds to findings that L3 expression can be observed as early as 8 *d.p.i* (Ledesma and Harrington 2011). The discovery of this gene is identical to that of the L3 activated collagen gene TC8100 found in *B. malayi* by Laney et al. (2010). These were the only target gene that met the assay criteria of non-amplification in gDNA or uninfected pools, and amplification in an L3 cDNA library. In

addition to detecting CK855471A, a novel constitutively expressed gene (*Ditph*), for all parasitic stages was detected in each sample to ensure the presence of parasite transcripts. This allows both transmission risks and xenomonitoring to be evaluated simultaneously (Laney et al., 2010).

This newly developed method has advantages over microscopic identification (Licitra et al., 2010), and standard PCR for parasite filarial DNA (Scoles and Kambhampati 1995). Standard PCR assays have been shown to exhibit increased sensitivity over dissection and can allow an increased number of samples to be analyzed simultaneously while reducing human error (Chambers et al., 2009; Fisher et al., 2007). Unfortunately, this technique misrepresents transmission potential because it detects DNA from all parasite stages in the mosquito and measures prevalence rather than infectivity. Also, using this technique non-competent mosquito vectors could be inaccurately identified as vectors. According to Fisher et al. (2007) parasite DNA can be detected in both vector and non-vector mosquitoes for two weeks or longer after they ingest Mf positive blood. A molecular assay that accurately identifies infectivity requires a method for preserving parasite RNA in mosquitoes, efficiently isolating and extracting parasite RNA from parasites in pools of mosquitoes, and identifying an L3-activated gene transcript to ensure only infective stage parasites are detected (Laney et al., 2008; Laney et al., 2010). The method described in this study meets these criteria.

Additional methods to detect *D. immitis* DNA include deep sequencing (Fu et al., 2013), high resolution melting (HRM) assay (Rojas et al., 2015), and nested PCR (Oi et al., 2015). The research outlined in this study along with current methodology provides a valuable tool for: 1) identifying mosquito vectors that are not already known in native

populations as well as those in different geographical regions, 2) clarifying the seasonality of transmission, 3) analysis of *D. immitis* gene regulation and 4) serving as a template for constructing parallel assays in other parasitic organisms.

In the future, work with this L3 detection assay could include sensitivity testing, specificity testing, and field studies. Sensitivity levels can be measured by mixing single infective individual mosquitoes (14 *d.p.i*) with uninfected mosquitoes in a variety of total pool sizes (e.g., 5, 10, 15, 20 and 25 mosquitoes). This test would determine sensitivity limits by determining the minimum and maximum number of mosquitoes needed in a pool to detect infectivity. Furthermore, specificity of the L3 activated gene can be tested against other infective vector species pools within the three major genera *Aedes*, *Anopheles* and *Culex* that are considered to be competent vectors including *Cx. pipiens* and *Ae. albopictus* in addition to performing tests against other filarial parasites in endemic areas such as *B. pahangi*, *B. malayi* (Laney et al., 2010) and *W. bancrofti* (Laney et al., 2008) which are close evolutionary species. For example, Laney et al. (2010) demonstrated that the *W. bancrofti* collagen gene CK85571A met the criteria of no gDNA amplification and PCR efficiency but amplified transcripts from infective *B. pahangi* pools and was considered unsuitable as an L3 activated gene in regions where the two parasites might be co-endemic. These tests are important for studies involving field-collected mosquitoes. Additional studies are needed to evaluate its efficacy for interpreting the effects of control efforts.

Dirofilaria immitis infections can be a public health concern as well as a major concern for animal health. Both canine and human dirofilariosis continue to be diagnosed within tropical, subtropical and temperate regions of the world (Vezzani et al., 2011;

Simón et al., 2012; Otranto et al., 2013). Little is known about the epidemiology and risk factors associated with human dirofilariosis (Dantas-Torres and Otranto 2013). Globally, most human cases of dirofilariosis are attributed to different *Dirofilaria* species (such as *D. repens*) rather than *D. immitis* (Simón et al., 2012). In the Americas, human dirofilariosis is primarily associated with *D. immitis* (Dantas-Torres and Otranto 2013). Several cases have been reported in North America including areas such as Texas and Florida (Theis 2005). This molecular assay demonstrates advancement in the "One Health" approach which embraces a cross-disciplinary collaborative effort between physicians, veterinarians, and scientists (including parasitologists and entomologists) to address diseases (e.g., rabies, Hendra virus, SARS, etc.) in which infection in one species can cause a disease in another (Gibbs 2014; Mellanby 2015). Understanding the biology of one species can help develop preventative approaches for another (Mellanby 2015). Considering the low frequency of documented human dirofilariosis cases reported actual occurrence could still be underestimated without advances in our ability to estimate transmission risks (Dantas-Torres and Otranto 2013). With a "One Health" approach the potential impact and benefits of both humans and animals will be considerable.

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APPENDIX A

Valdosta State University Biosafety Committee Approval Form

VALDOSTA STATE UNIVERSITY

MEMORANDUM OF UNDERSTANDING

AND

AGREEMENT (MUA) FOR BIOHAZARDS OTHER THAN RECOMBINANT DNA EXPERIMENTS

DATE: 16 March 2013

RESEARCHER'S
NAME Eric W. Chambers

RESEARCHER'S TITLE Assistant Professor

PHONE NO. 249-2736

DEPARTMENT Biology

BUILDING. & ROOM NO(s) BSC 2042

GRANTING AGENCY VSU

GRANT NO. (IF APPLICABLE)

TITLE OF GRANT OR PROJECT:

Development of an RT-PCR assay for the Detection of *Dirofilaria immitis* L3 larvae: A Tool for Understanding Dog Heartworm Transmission in Canines.

A. Describe the experiments involving biohazard(s). Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it.

This project will involve the development of a reverse-transcriptase (RT-PCR) assay for the detection of L3 stage larvae from the filarial parasite *Dirofilaria immitis*. This parasite is the causative agent of dog heartworm. Mosquitoes infected with *D. immitis* will be used in a time course study to identify stage specific expression of a *D. immitis* target gene.

We propose the following experiment along with an alternative experiment.

Primary Experiment

1. ***Aedes aegypti* mosquitoes infected with the Missouri 2005 strain of *D. immitis* provided to us by the NIAID/NIH Filariasis Research Reagent Resource Center (FR3). The FR3 is located on the campus of the University of Georgia and serves as a repository for reagents and supplies needed for work with filarial worms.**

2. **Infected mosquitoes will be held in the Adaptis-A100 environmental chamber (Conviron, Pembina, ND, USA) at 28°C ± 2°C at 80% relative humidity.**

3. **Ten infected mosquitoes will be tested for candidate gene expression at 2,4,6,8,10 and 12 days.**

4. **Five mosquitoes will be dissected at each time point to monitor parasite development. The other five mosquitoes at each time point will be pooled together and RNA will be extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA).**

5. **A second group of uninfected mosquitoes will also be tested at each time point and will serve as negative controls.**

6. **Three replicates of this design will be performed.**

7. **Following identification of the L3 specific target gene we will determine the sensitivity of our primer set by combining a putatively *D. immitis* infected mosquitoes with unfed control mosquitoes in pool sizes of 10, 15, and 20 mosquitoes.**

8. **RNA will be extracted from these mixed cohorts as described above and then screened for the presence of infective L3 stage *D. immitis* larvae.**

Alternative experiment

If mosquitoes infected in the FR3 cannot survive the shipping process or if the logistics of shipping infected mosquitoes proves untenable we propose the following alternative experiment.

1. **Blood from a dog infected with the Missouri 2005 strain of *D. immitis* (10-20 cc) will be shipped to Valdosta State University from the FR3 campus at UGA.**

2. **Infected dog blood will be diluted with bovine blood (Hemostat, Dixon, CA USA) in sodium citrate to a concentration of 10 mf/ml.**

3. **Female *Aedes albopictus* mosquitoes (5-7 days old) will be allowed to feed to repletion on infected blood using either a Hemotek system feeder (Discovery Workshops, Accrington, UK, hemotek@discoveryworkshops.co.uk) or a modified *Mishra* feeder where parafilm is stretched over the outside of a container filled with warm water and a small amount of blood is injected into the space between the parafilm and the container.**

4. **Mosquitoes are then held in the Adaptis-A100 environmental chamber and cohorts of 10 mosquitoes are removed and analyzed as described in steps 3-8 of the 1st proposed experiment.**

MUA FOR BIOHAZARDS OTHER THAN RECOMBINANT DNA EXPERIMENT
(CONTINUED)

B. Assess the levels of physical containment required for the experiments.

Maintenance and blood feeding of mosquitoes will occur within an Adaptis A100 Reach-in environmental chamber. This environmental chamber maintains a constant environment (temperature and humidity) for *Aedine* mosquitoes.

Because *D. immitis* is NOT a human pathogen and because the mosquito species we will be working with will be either 1) already present in our geographical area or 2) unable to establish themselves permanently in our area, we are only required to hold our mosquitoes at Arthropod Containment Level-1 (ACL-1). Nevertheless our lab will follow when possible, Arthropod Containment Level-2 (ACL-2) practices to ensure that no accidental releases occur.

The following guidelines are adapted from "Arthropod Containment Levels (ACLs), VECTOR-BORNE AND ZOONOTIC DISEASES Volume 3, Number 2, 2003.

1. **Mosquitoes will be held in a specialized environmental chamber out of the flow of traffic in order to prevent accidental contact and release by laboratorians, custodians, and service persons.**

2. **The lab is maintained so as to enhance detection of escaped arthropods. Items that could provide harborage for arthropods are removed. Doors and drawers are kept closed. Insect food is kept in sealed containers.**

3. **Cages and containers are cleaned with disinfectant and/or autoclaved.**

4. **Cages to hold mosquitoes are non-breakable and screened with mesh to prevent escape. Our lab utilizes collapsible cages manufactured by Bioquip Products (Rancho Dominguez, CA) and designed for use in vector control research labs.**

5. **Arthropod materials will be autoclaved and infected arthropods not used for RNA or DNA extraction will be frozen and then autoclaved.**

6. **Arthropods will be identified properly with species, strain, investigator name, date, etc. Labels will be attached firmly to container.**

7. **Escaped arthropod monitoring: traps will be set up in lab to capture escaped arthropods. Investigators will actively seek and capture visually identified escaped arthropods. Infected arthropods will be captured using a battery powered vacuum aspirator.**

8. **Records of exterior captures will be maintained.**

9. **Lab personnel will wear lab coats and gloves when working with infected mosquitoes and/or blood.**

10. **Signage will be used that will notify all persons entering the area that arthropod vectors are present. When infected mosquitoes are present a BSL-1 biohazard sign will be posted on the entrance to the lab and on the entrance to the environmental chamber.**

11. **The lab and the environmental chamber will be locked at all times. Access is only made avail to the Principal Investigator and lab staff.**

C. Describe the facilities and specific procedures that will be used to provide the required levels of containment.
-Page 3-

Additional guidelines that the staff will follow to ensure proper containment are found in

"Arthropod Containment Levels (ACLs), VECTOR-BORNE AND ZOONOTIC DISEASES Volume 3, Number 2, 2003.

http://online.liebertpub.com/doi/pdf/10.1089/153036603322163475

D. Describe the procedures and precautions to be followed if biohazardous organisms or agents are to be transported between laboratories.

Shipping of infectious materials will be conducted by the FR3. The FR3 is contracted by the NIH to provide these needed materials to researchers throughout the world. There is no cost for the reagents although the researcher must pay for the shipping. FR3 will prepare and package the materials following state and federal guidelines. Materials are shipped via Fed Ex. A Material Transfer Agreement will need to be signed between Valdosta state University and FR3 prior to shipment of materials.

Upon arrival the Principal Investigator, Dr. Chambers, will assume responsibility of the materials and transfer either mosquitoes or blood to their proper holding location within his lab.

MUA FOR BIOHAZARDS OTHER THAN RECOMBINANT DNA EXPERIMENTS (CONTIUED)

E. The undersigned agree to certify the following conditions of the proposed research:

1. The information above is accurate and complete. We agree to accept responsibility for training of all laboratory workers involved in the project. We agree to comply with the CDC/NIH/USDA/EPA requirements pertaining to shipment and transfer of hazardous biological materials. We are familiar with and agree to abide by the provisions of Valdosta State University Biosafety Manual, which outlines standards for conducting experiments with bio hazardous agents.
2. We understand that only the organisms specified are covered by this MUA, and that work with other organisms or types of biohazards may require other MUAs.

4-17-2013

Principal Investigator — Date

4-17-13

Department Head — Date

* *
* *

F. The VSU Committee on Biosafety has determined, based on information provided by the principal investigator, that:

✓ (a) No special medical surveillance (other than usual University health programs) is required for the project described in this MUA;

_____(b) The following specific medical surveillance procedures must be carried out, for the

individuals listed by name, before commencing the project described in this MUA:

G. We certify that the Valdosta State University Committee on Biosafety has reviewed the proposed project and has found it to be in compliance with the VSU Biosafety Manual, which outlines standards for conducting experiments with biohazardous agents.

[signature] 4/17/13

Chairperson, VSU Committee on Biosafety DATE

[signature] 4/24/2013

VSU Biosafety Officer

APPENDIX B

Molecular Genetics of *Dirofilaria immitis* Biosafety Manual

Biosafety Level 2

Personal Protective measures

I. Personal Protection

1. Lab personnel will wear gloves and eye protection when working with biological material (including infectious agents).
2. Lab personnel will wear appropriate foot-wear (closed-toe shoes) when working in the lab.
3. Lab personnel will inform the principal investigator (Dr. Eric Chambers) if they are injured while working in the lab.
4. A first aid kit is available in the lab for minor cuts and wounds. Lab personnel should still inform the principal investigator even for minor injuries.
5. Lab personnel will be trained in the use of the chemical eye-wash station and the chemical shower station.
6. Lab personnel will be trained in use of the chemical spill kit.

Decontamination Procedures

I. Decontamination of work surfaces

1. Work surfaces will be treated with 10% sodium hypochlorite for 2 minutes prior to initiation of work and following all work with infectious agents.
2. Surfaces will then be wiped with paper towels.
3. Lab personnel will wear gloves while carrying out decontamination protocols.

II. Spills of potentially hazardous materials or hazardous waste (from VSU biohazard manual)

General Principles

1. Primary responsibility for preventing or/and containing and cleaning up laboratory spills remains with the principal investigator (Dr. Eric Chambers) or laboratory supervisor (Dr. Eric Chambers). Laboratory protocols should be carefully designed to prevent biological, chemical and/or radiation spills.
2. When accidents occur that involve the mishandling or escape of biohazardous materials, the principal investigator or laboratory supervisor is to be notified immediately. Spills of high-risk organisms (certain Class 2 and all Class 3) should be reported to the Biosafety Officer during normal working hours or to the Valdosta St ate University Public Safety Division at the emergency telephone number after normal working hours by the principal investigator or laboratory supervisor. The Public Safety Division will contact the Biosafety Officer for appropriate response. All employees and/or students have an obligation to themselves and their colleagues to report accidents immediately in order to minimize potential hazard.
3. When a biohazardous spill also involves radioactivity, cleanup procedures may have to be modified. The extent of the modification will depend on the level of radiation and the nature of the isotope involved. The Radiation Safety Officer should be called during normal working hours, or the Valdosta State University Public Safety Division should be called after normal working hours.

4. The attached guidelines are intended to assist the principal investigator, laboratory supervisor, and other responsible individuals who may be involved in the cleanup of biological spills.

Biohazardous Spills Inside Laminar Flow Biological Safety Cabinets (LFBSC)

The occurrence of a spill in a biological safety cabinet poses less of a problem than a spill in an open laboratory as long as the spilled materials is contained in the biological safety cabinet. Decontamination of the work zone can usually be effected by direct application of concentrated liquid disinfectants along with a thorough wipe down procedure. Gaseous decontamination may be required to clean up the interior sections of the cabinet.

Procedures for Decontamination of LFBSC

1. Chemical decontamination procedures should be initiated immediately while the biological safety cabinet continues to operate. Continuing the operation of the LFBSC helps to prevent the escape of contaminants from the cabinet.
2. Wearing protective gloves spray or wipe walls, work surfaces, and equipment with an appropriate decontaminating solution. A disinfectant detergent, such as Wescodyne or Environ has the advantage of detergent action on extraneous organic substances, which may interfere with the microbicidal activity of the disinfectant.
3. Flood tray top, drain pans, and catch basins below work surface with decontaminating solution and allow to stand for 20 minutes.
4. Drain excess decontaminating solution from tray and drain pans into cabinet base. Lift out tray and removable exhaust grille work. Clean the top and bottom (underside) surfaces using a sponge or clean cloth soaked in decontaminant solution. Following the cleaning process, replace the tray and grille work in their proper position. Place gloves and sponge or cloth in autoclave pan and autoclave these items.
5. Drain decontaminating solution from cabinet base into appropriate container and autoclave according to standard procedures.
6. If gaseous decontamination of the cabinet's interior sections is needed, call the Biosafety Officer.

Biohazardous Spills Outside Laminar Flow Biological Safety Cabinets

The protocol to be used in cleaning up of spills involving microorganisms will depend on the amount of material spilled and the degree of laboratory containment required. If individuals believe that their outer garments have been contaminated, they should remove their clothing in the laboratory area and place them in an autoclave or a container for autoclaving. They should change into clean clothing in a non-contaminated area. All laboratory personnel should keep a complete change of clothing, including shoes at the laboratory in case of spills. Special care in decontamination may be necessary if a spill goes under or between fixed furniture or behind base moldings (floor/wall) or if floor penetrations are involved.

Minor Spills (less than 10 ml and generating little aerosol) on equipment, laboratory benches, walls, or floors:

1. Wipe up the spill with the soaked paper towels and place the used towels in an autoclave pan and autoclave.
2. Pour decontaminating solution around and on the area of the spill. Let stand for 20 minutes then wipe up with paper towels. Place gloves and paper towels in autoclave pan and autoclave.
3. Wash hands and other apparently contaminated areas again with soap and water.

Major Spills (more than 10 ml or with considerable aerosol):

1. Close laboratory doors and post warning signs to prevent others from entering the laboratory.
2. Wash hands and other apparently contaminated areas with soap and water.
3. Report the accident to the Supervisor and to the Biosafety Officer.
4. If personal clothing is contaminated, remove all outer clothing and place it in autoclave or container for autoclaving. Put on clean garments.
5. Leave the laboratory for 20 minutes to allow dissipation of aerosols created by the spill.

6. Upon returning to the laboratory to start decontamination, check to see if laboratory doors are closed and appropriate signs are displayed. Put on surgical gloves. Respirators or other safety equipment may be required, depending on the microorganism involved. Check with the Principal Investigator or Laboratory Supervisor or Biosafety Officer.
7. Pour a decontamination solution around the spill and allow this solution to flow into the spill. Paper towels soaked with decontamination solution may be used to cover the area. Do not pour decontamination solution directly onto the spill in order to avoid additional release of aerosols.
8. Let decontamination solution – microorganism mixture stand for 20 minutes or longer to allow adequate contact time.
9. Using autoclave dust pan and squeegee transfer all contaminated materials to deep autoclave pan, cover with suitable cover, and autoclave according to standard directions.
10. Place dustpan and squeegee in an autoclavable bag and autoclave according to standard directions.
11. Remove gloves and other contaminated garments and place them in an autoclave container for autoclaving.
12. Thoroughly wash hands, face, and other apparently contaminated areas.

Liquid Disinfectants

Laboratory personnel should be familiar with the various disinfectants that will effectively kills the biohazardous agents being used. The following information is provided to assist in your selection of appropriate disinfectants. Alcohols– Ethyl and Isopropyl are good disinfectants for the vegetative forms of bacteria and enveloped viruses.

Ethyl Alcohol

1. Use Dilution: 70-95%
2. Inactivates: vegetative bacteria and enveloped viruses, has variable results with non-enveloped viruses and is ineffective with bacterial spores.

3. Other Characteristics: flammable, eye irritant, and toxic [Threshold limit value (TLV) – 1000 ppm]

Isopropyl Alcohol

1. Same as for Ethyl Alcohol except the TLV = 400 ppm.

Chlorine Compounds– The germicidal effect of chlorine compounds is dependent upon the release of hypochlorous acid and is therefore dependent upon the available chlorine.

1. Allow a contact time of from 10 to 30 minutes.
2. Use Dilution: 500 ppm available chlorine is recommended for vegetative bacteria and most viruses.
3. Chlorine solutions that are neutral or slightly acidic and with a concentration of approximately 2500 ppm are needed for effectiveness against bacterial spores. Undiluted common household bleach (Clorox)is alkaline with a pH of 8. or greater. Household bleach typically contains 5.25% sodium hypochlorite for 52500 ppm available chlorine.
4. Other Characteristics: Chlorine compounds are corrosive to metals; leave a residue; irritate the skin, eyes, and respiratory tract, and are toxic. Chlorine compounds are also rapidly inactivated by organic matter. While chlorine compounds are not generally recommended for routine use, undiluted household bleach is frequently used with biological spills.

Iodophors - The germicidal effect of iodophors is dependent on the free iodine released from the compound in which it is contained. Allow a contact time of 10 to 30 minutes.

1. Use Dilution: 25 to 1600 ppm of available iodine. Solutions containing 75 to 150 ppm are generally recommended.
2. Inactivates: vegetative bacteria, fungi and viruses. There is poor activity against bacterial spores.
3. Other Characteristics: Although iodophors are less harmful to man than chlorine compounds they can irritate the skin and eyes. Iodophors are corrosive (less than chlorine), they leave a residue and may stain. Iodophor stains, however, can be readily removed with solutions of sodium thiosulfate ($Na2S2O3$). As with the chlorine compounds, iodophors are rapidly inactivated by organic matter. One advantage is that iodophors have a built-in indicator. As long as the solution is brown or yellow it is still active.

Phenolic Compounds - These are effective against vegetative bacteria (including Mycobacterium tuberculosis), fungi, and enveloped viruses. Effectiveness against non-enveloped viruses is variable depending on the virus. The phenols are ineffective against bacterial spores.

1. Use Dilutions: 1.0 – 5.0% Solutions containing 0.5 – 2.0% phenol are effective against enveloped viruses.
2. Other Characteristics: Phenols are corrosive and may leave a sticky, gummy residue.
3. Phenolic compounds are irritating to the skin and eyes and are relatively toxic – Phenol
4. TLV for skin is 5 ppm.

Quaternary Ammonium Compounds – The efficacy of Quaternary Ammonium compounds still generates considerable controversy. Quats are effective in destroying ordinary vegetative bacteria and lipid containing virus but are not effective against Pseudomonas, Proteusand other gram-negative bacilli. Also, Quats are not effective against bacterial spores at the usual use concentrations of 1:750.

1. Use Dilutions: 0.1 to 2.0%
2. Other Characteristics: Quats are surface-active compounds which posses the useful property of lowering the surface tension of the solution.
3. Other advantages include being nontoxic, odorless, nonstaining, noncorrosive to metals and stable. If used at recommended concentrations, Quats are nonirritating.
4. Quaternary Ammonium compounds are rapidly inactivated by organic matter.

Occupational Health and Medical Surveillance of Human Dirofilariasis caused by *Dirofilaria immitis*

I. Background

Human dirofilariasis is caused by infection with parasitic nematodes worms primarily from three species within the genus *Dirofilaria*; *D. immitis*, *D. repens*, and *D. tenuis*. Dirofilariasis is primarily a disease of domestic and wild animals. On rare occasions humans can be a spillover host, thus it us technically a zoonotic pathogen.

II. Transmission

Dirofilariasis requires transmission through an obligate mosquito vector. Dirofilariasis cannot be transmitted person-to-person nor can it be transmitted from humans to a mosquito and then back to humans. Because we will not be working with live infected mosquitoes in our facility, **it is impossible for disease transmission to occur.**

III. Symptoms

Human infections with *D. immitis* are can result in inflammation within the pulmonary arteries. These areas of inflammation often appear as coin lesions on x-rays. Human infection with *D. immitis* is most often asymptomatic but when symptoms do appear they include cough (can be a bloody cough), chest pain, and pleural effusion.

IV. Medical Surveillance

Personnel working with the frozen mosquitoes will be briefed on the pathogen *D. immitis*. All personnel will be instructed to tell their supervisor (Dr. Eric Chambers, if they are experiencing any of the symptoms described in part III). They will be instructed to relate their work history to physicians to avoid misdiagnosis of coin lesions (if such are observed on chest x-rays).

V. Treatment

No vaccine exists for dirofilariasis and in many cases no treatment with medicines is required. Surgical removal of lung granulomas is the definitive treatment.

APPENDIX C

Isolation of Total RNA from Tissue Samples

Isolation of Total RNA from Tissue Samples

Work under RNase-free conditions.

This protocol is based on the Invitrogen protocol for total RNA isolation from any tissue sample using TRIzol LS reagent, and has been adapted for mosquito midgut tissue.

Reagents required:

-TRIzol LS Reagent (Invitrogen #10296-010)

-Chloroform (without any additives, such as isoamyl alcohol)

-Isopropyl alcohol

-75% ethanol and 100% ethanol (in DEPC-treated water)

-RNase-free water (DEPC-treated water)

-RNase inhibitor (40 units/µL, Invitrogen #10777-019)

Homogenization

Grind mosquito tissue (up to 30 adults or 60 midguts) using a blue pestle in a 1.5 mL tube with:

250µl RNase-free water

750µl TRIzol LS Reagent

Incubate for 10 min at room temperature to permit the complete dissociation of nucleoprotein complexes.

Phase Separation

1. Add 200 µL of chloroform, shake tubes vigorously by hand for 15 s, and incubate at room temperature for 10 min.
2. Centrifuge at 11,000 rpm (10,000 *g*) for 15 min at 4 °C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 70% of the volume of TRIzol used for homogenization.

RNA Precipitation

1. Transfer the aqueous phase to a sterile tube containing 1 µL of RNase inhibitor (you can save the organic phase if isolation of DNA or protein is desired). Optional: to facilitate isolation of RNA from small quantities of sample, add 10-

40 µg of RNase-free glycogen (Invitrogen #10814-010) as a carrier to the aqueous phase.

2. Precipitate the RNA from the aqueous phase by adding 600 µL of cold isopropyl alcohol. Invert to mix, and incubate the samples for 15 min at room temperature.
3. Centrifuge at 11,000 rpm for 10 min at 4 ºC. The RNA precipitate should be visible as a gel-like pellet on the side and bottom of the tube.

RNA Wash

1. Remove the supernatant.
2. Wash the RNA pellet with 1 mL of cold 75% ethanol. Mix the sample by gentle vortexing.
3. Centrifuge at 9,000 rpm (7,000 x *g*) for 5 min at 4 °C.
4. Wash the RNA pellet another time with 500 µL of cold 100% ethanol.
5. Centrifuge at 9,000 rpm for 2 min at 4 ºC.

RNA Resuspension

1. Remove the ethanol with a pipet and air-dry the RNA pellet for 5-10 min. Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/A280 ratio <1.6.
2. Dissolve the RNA in RNase-free TE, pH 8.0 (10-100 µL, depending on amount of RNA) by incubating for 10 min at 60 ºC. Add 1 µL RNase inhibitor for long-term storage at -80 ºC.