Identification of Excreted-Secreted Antigens of Filarial Nematodes to Develop Diagnostic Methods

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Abstract

The identification of excreted-secreted (ES) proteins of filarial nematodes as potential diagnostic reagents is an important requirement for the development of methods to determine level of infection in the host, especially for human filariae. *Dirofilaria immitis*, the canine heartworm, is a widespread and important veterinary pathogen and is a useful model for filarial parasites of humans. An analysis of proteins released from adult *D. immitis* (the secretome) in culture is available. We sought to identify *D. immitis* ES proteins found *in vivo* to validate the *in vitro* secretome and to investigate them as potential diagnostic reagents. Cultures of *D. immitis* adults obtained from infected dogs were maintained for 72 hr with daily changes of media. Proteins were concentrated from spent media by standard methods and were passed through Protein-A columns containing purified IgG antibodies from heartworm-infected dogs. Following extensive washing, heartworm proteins recognized by the antibodies were eluted from these columns and submitted for analysis by tandem mass-spectrometry (MS/MS). As a comparison, somatic proteins from adult *D. immitis* female parasites and microfilariae were also processed and analyzed by the same protocol. Six, nine and twelve proteins were identified by MS/MS in the ES, adult female, and microfilariae samples, respectively. The identification of the most abundantly secreted parasite proteins present in the serum of infected hosts offers a rational approach to the development of new diagnostic assays that may be applicable across the Filarioidea.
Résumé

L'identification des protéines excrétées-sécrétées (ES) des nématodes filaires comme de potentiels réactifs à des fins diagnostiques est un prérequis important pour le développement de méthodes visant à déterminer le niveau d'infection chez l'hôte, particulièrement dans le cas de filaires humains. Dirofilaria immitis, le ver du cœur canin, est un important pathogène vétérinaire répandu et est un modèle utile pour étudier les filaires parasitaires chez les humains. Une analyse des protéines sécrétées par des D. immitis adultes (le sécrétome) en culture in vitro est disponible. Nous cherchions à identifier des protéines ES de D. immitis présentes in vivo pour valider le sécrétome in vitro et pour les considérer comme de potentiels réactifs à des fins diagnostiques. Des cultures de D. immitis adultes obtenues de chiens infectés furent maintenues pendant 72 heures en changeant le milieu de culture quotidiennement. Les protéines contenues dans le milieu usé furent concentrées par des méthodes conventionnelles et furent passées à travers des colonnes Protein-A contenant des anticorps IgG purifiés de chiens infectés avec des vers du cœur. Suivant un rinçage rigoureux, les protéines des vers reconnues par les anticorps furent éluées et soumises à une analyse par spectrométrie de masse en tandem (MS/MS). À titre comparatif, les protéines somatiques de parasites D. immitis adultes femelles et microfilaires furent soumises au même processus et analysées suivant un protocole identique. Six, neuf et douze protéines furent identifiées par MS/MS dans les échantillons d'ES, des adultes femelles et des microfilaires, respectivement. L'identification des protéines du parasite les plus
abondamment sécrétées présentes dans le sérum des hôtes infectés représente une approche rationnelle au développement de nouveaux tests diagnostiques qui pourront être applicable aux Filarioidea en général.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANNEX</td>
<td>Annotation Expander</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
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<tr>
<td>C18</td>
<td>Stage Tip C18</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CRCHUQ</td>
<td>Centre Hospitalier Universitaire de Québec</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DSS</td>
<td>Disuccinimidyl Suberate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ES</td>
<td>Excreted-secreted</td>
</tr>
<tr>
<td>ES MS/MS</td>
<td>Electrospray Mass-Spectrometry</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FR3</td>
<td>Filariaasis Research Reagent Resource Center</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full Width at Half Maximum</td>
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<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>x g</td>
<td>Times Gravity</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>hr</td>
<td>Hour</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>IVM</td>
<td>Ivermectin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>L2</td>
<td>Second larval stage</td>
</tr>
<tr>
<td>L3</td>
<td>Third larval stage</td>
</tr>
<tr>
<td>L4</td>
<td>Fourth larval stage</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MF</td>
<td>Microfilaria</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
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<td>mm</td>
<td>Millimeter</td>
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</table>
mM  Millimolar
MS   Mass-spectrometry
MS/MS  Tandem Mass-spectrometry
M.Sc.  Master of Science
MVB  Multivesicular Bodies
MW  Molecular weight
MWCO  Molecular Weight Cut Off
μg  Micrograms
μL  Microliters
μM  Micromolar
nanoLC  Nanoscale Capillary Liquid Chromatography
N₂  Nitrogen Gas
NaCl  Sodium Chloride
NCBI  National Center for Biotechnology Information
NIH  National Institutes of Health
nm  Nanometers
NP-40  Nonyl phenoxyethoxylethanol Detergent
PBS  Phosphate buffered saline
pH  Power of Hydrogen
QqTOF  Quadrupole time-of-flight
RNA  Ribonucleic acid
RP  Reverse-phase
RPMI  Roswell Park Memorial Institute
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA  Trichloroacetic acid
TFA  Trifluoroacetic acid
Th1  Helper T cell response 1
Th2  Helper T cell response 2
Tris  Trisaminomethane
USA  United States of America
WHO  World Health Organization
w/v  Weight per Volume
List of Tables:

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**Figure 2.** Current geographical distribution of canine dirofilariasis. Blue, *Dirofilaria immitis* infections; green, *Dirofilaria repens* infections; orange, presence of both species (Simon *et al*., 2012).

**Figure 3.** The adult excretory-secretory system in *Caenorhabditis elegans*. Lateral oblique view. The excretory cell (red), the gland cell (blue) and the duct cell (tan) are all joined together at a specialized intercellular junction called the excretory-secretory junction, where excreted materials are passed into the duct to be transported through the pore cell (yellow) to the outside. In this view, excretory and duct cell bodies are removed to reveal the junction. (http://www.wormatlas.org)

**Figure 4.** Crosslinking canine antibodies to Protein-A column and immunogenic protein purification. Step 1. Canine antibodies are bound and crosslinked to Protein-A beads in an affinity column. Step 2. Protein sample is added to the column where immunogenic proteins are recognized by bound antibodies. Step 3. Unbound non-immunogenic proteins are washed away. Step 4. Immunogenic proteins are collected during elution.
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**Figure 5.b** Silver stained 4-12% gradient polyacrylamide gel. Lane 1 contains ES proteins post-Protein-A column, lane 2 is Benchmark Prestained ladder.

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**Figure 8.** Distribution of Gene Ontology terms (Molecular Function - level 2) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.

**Figure 9.** Distribution of Gene Ontology terms (Molecular Function - level 4) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.

**Figure 10.** Distribution of Gene Ontology terms (Biological Process - level 2) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.

**Figure 11.** Distribution of Gene Ontology terms (Biological Process - level 3) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.
CHAPTER I

INTRODUCTION
**Introduction**

**Dirofilaria**

Dirofilaria is the parasitic disease caused by either *Dirofilaria immitis* or *Dirofilaria repens*. *D. immitis* is a filarial nematode that can cause heartworm infection primarily in dogs; it has a nearly worldwide distribution including North and South America, Asia, and parts of Europe (McCall *et al.*, 2008). *D. immitis* has a life cycle of about 7-9 months that begins when a mosquito ingests microfilariae (MF) during a blood meal from an infected host (Figure 1). Approximately 24 hr post-ingestion, the MF migrate from the midgut of the mosquito to the Malpighian tubules where they remain for 6-7 days. The larvae then travel to the lumen of the tubules to molt into the second larval stage (L2). Within 2-3 days, they molt again to the infective third stage (L3) and migrate to the mouthparts of the mosquito (McCall *et al.*, 2008; Morchon *et al.*, 2012). The infective L3 are transmitted to a new host when the mosquito takes a blood meal. In 3-12 days after initial infection, the L3 molt and become fourth stage (L4) larvae. The L4 migrate through the body of the host, attempting to reach the heart. A final molt to the adult stage occurs between day 50-70; the adults will reach the heart of their host between day 90-120. When the adults reach the heart, they are 2-4 cm in length; upon further development, the female worms will increase in length up to ten-fold. Hosts typically develop a patent infection marked by the presence of MF in the bloodstream approximately 7-9 months post-infection (McCall *et al.*, 2008).
Figure 1. During a blood meal, an infected mosquito introduces L3 *D. immitis* larvae to the canine host (1). In the host, the L3 larvae molt into L4 and then adults. The adults reside in pulmonary arteries and right ventricle of the heart (2). In the pulmonary arteries and heart, female worms produce microfilariae over their lifespan (3). The microfilariae are primarily found in peripheral blood where a mosquito can ingest them during a blood meal (4). After ingestion, the microfilariae migrate into the mosquito’s Malpighian tubules in the abdomen (5). Microfilariae then develop into L1 larvae (6) and subsequently into L3 larvae (7). The third-stage infective larvae then migrate to the mosquito’s proboscis, and the life cycle begins again (8). (CDC, 2012)

*Dirofilaria immitis* Distribution

*D. immitis* has been identified in over 30 host species, including dogs, cats, monkeys, marine mammals, and rodents; it is by far most prevalently found
in the dog. Dogs are one of the most popular domesticated animals and are frequently kept as pets. Domesticated dogs throughout the world commonly travel with their owners and can easily transmit the parasite to other susceptible animals. This most likely explains why there are few places left where heartworm infection is rarely seen (Figure 2) (McCall et al., 2008; Simon et al., 2007).

Figure 2. Current geographical distribution of canine dirofilariasis. Blue, *Dirofilaria immitis* infections; green, *Dirofilaria repens* infections; orange, presence of both species (Simon et al., 2012).

Pathology of Dirofilariasis

Pathology associated with *D. immitis* infection can range from a completely asymptomatic state to difficulty with movement, exercise intolerance, lethargy, lung damage, and, in dogs that have high adult worm burdens, death.
The most common and successful control method available is monthly administration of a macrocyclic lactone anthelmintic such as Ivermectin (IVM). This kills the L3 larvae, effectively preventing them from molting into L4, and the L4 larvae (Bowman et al., 2011). Treatments that specifically target adult *D. immitis* worms are also utilized. Arsenic-based compounds can be administered to kill adult worms, but these drugs pose a substantial risk to the host’s health. Monthly administration of macrocyclic lactones such as IVM, milbemycin oxime, moxidectin, and selamectin are commonly used as prophylactics in dogs, while drugs such as doxycycline, which target the endosymbiont *Wolbachia*, can be administered for 4-6 weeks every 3-4 months. Combining IVM and doxycycline has been found to be helpful in treating adult worms; however, these drugs must be administered together at standard doses for a year or more to have adulticidal effects (Grandi et al., 2010). The only adulticidal drug currently permitted by the US. Food and Drug Administration (FDA) is melarsomine dihydrochloride which is administered via deep intramuscular injection. In some cases, surgical removal of the adult worms can also be attempted, but this is only a temporary solution, as many worms are in areas that are not easily accessible (Bowman et al., 2011; McCall et al., 2008; Nelson et al., 2005).

**Detection of *Dirofilaria immitis***

Detection of *D. immitis* in the host can be accomplished through examination of a blood sample. MF can be detected in direct blood smears or
using a modified Knott’s test, while Millipore filtration systems can be utilized to detect MF in blood samples. Unfortunately, the quantity of MF present in a blood sample does not correlate with the quantity of adult parasites present in the host. Consequently, methods that rely solely on MF detection in the blood are not effective indicators of disease severity or potential severity. A more common method currently used to detect the presence of *D. immitis* in the host is an antigen test. Enzyme-linked immunosorbent assay (ELISA), lateral flow, and immunomigration techniques can be utilized as immunodiagnostics for detection of *D. immitis* antigens in serum. Although tests currently available are highly sensitive, the antigens released are not well characterized in the literature. Many of the diagnostic methods utilize antigens that are only present in the host after the adult worms are present, making the tests ineffective during the first 7-9 months post-infection (Ettinger *et al.*, 2010; Nelson *et al.*, 2005).

**Wolbachia Endosymbiont**

An important aspect of infection by several filarial nematodes, including *D. immitis*, is the presence of an endosymbiont intracellular α-proteobacteria known as *Wolbachia*. Typically, *Wolbachia* is found in the oocytes, ovaries, hypodermis, and developing embryos of female worms and in the lateral chords in adult worms. While it appears that *Wolbachia* is essential to filarial growth, development, and survival, the symbiotic relationship and interactions between the two organisms are not completely understood (Slatko *et al.*, 2010; Taylor *et
Wolbachia also appears to be a driver of inflammation within the host via the innate immune response; in addition, Wolbachia promotes the activation of neutrophils and has the ability to classically activate macrophages. Nematode infections characteristically promote strong Th2 responses in the host; it is thought that Wolbachia aids the parasite by encouraging a Th1 immune response which can lead to endothelial proliferation and dilation of blood vessels that contribute to the chronic disease state (Taylor et al., 2005; Taylor et al., 2010).

The endosymbiotic relationship of D. immitis and Wolbachia is demonstrated during the initial stages of the host immune response following infection with D. immitis. Wolbachia promotes the characteristic Th1 response in the host which allows D. immitis to subvert the normal Th2 immune response, promoting inflammation and dilation of the host’s blood vessels, which provides easier access to the peripheral blood for the parasites (Frank et al., 2010; Simon et al., 2007; Slatko et al., 2010).

**Excreted-Secreted Proteins from Filarial Parasites**

Filarial nematodes, like many other parasites, have excretory and secretory processes that are important in altering the host’s immune response and sponsoring parasite survival (Harnett et al., 2008). These excreted or secreted (ES) factors, typically proteins, have many roles, including evasion of host immunoregulatory responses, permeation of tissue barriers, and the initial penetration of host cells and tissues (Knox, 2000). The ability of the parasite to
alter the production of ES proteins to efficiently adapt to its environment enhances the parasite’s ability to survive within the host. ES proteins have a profound impact on the host immune response to infection by the parasite. In most cases, the immune response is modulated by either activation or upregulation of immune system factors or, conversely, inhibition of immune system factors; such modulations enhance the ability of the worms to survive and thrive within the host. In nematode parasite infections, disease development tends to be quite slow and parasitism affects the host in a cumulative fashion by gradually increasing the pathology of disease and consistently inducing adverse immune responses and reactions (Maizels et al., 1991).

ES proteins, including proteinases, cystatins, aspins and serpins, lectins, antioxidant enzymes, immunomodulatory components (including complement binding factors and immunosuppresants), and nucleotide metabolizing enzymes from several parasitic nematodes have been identified. These secreted products were identified by proteomic methods and functional assays that measure the enzymatic activity of ES factors (Dzik, 2006; Hewitson et al., 2009). Proteomic methods such as mass-spectrometry coupled with both genome and transcriptome mapping have resulted in the identification of ES proteins released by Brugia malayi and Heligmosomoides polygyrus in vitro (Bennuru et al., 2011; Hewitson et al., 2008; Moreno et al., 2008; Moreno et al., 2011). ES proteins have also been isolated and characterized for Meloidogyne incognita, a parasitic plant nematode; Ancylostoma caninum, the canine hookworm; and Strongyloides stercoralis, the threadworm (Bellaﬁore et al., 2008; Mulvenna et al., 2009; Soblik et al., 2011). Not all of these nematode datasets were compared against the
entire genome or transcriptome of their respective organism. Despite this incomplete comparison, many ES proteins have been found in the secretomes of the parasites examined, and the assignments have been quite good overall (Geary et al., 2012).

**Identification of *D. immitis* in vitro Secretome**

A recent study by Geary et al. 2012 examined adult *D. immitis* to determine which ES proteins are secreted in the highest abundance by the parasite in culture. While current detection methods utilized for filarial nematode infection generally rely on poorly characterized antibodies or antigen, identification of specific ES proteins could lead to the development of new immunodiagnostic tests and procedures that could more accurately indicate worm burden in the host. In addition, new treatment strategies and vaccines that would unambiguously target these ES proteins could also be developed (Blaxter, 2003; Geary et al., 2012; Gioia et al., 2010).

In this study, adult *D. immitis* were removed from their canine hosts and placed in culture fluid to collect proteins secreted *in vitro*. The secreted proteins were then pelleted and run on a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel before the proteins were sliced from the gel and submitted for MS/MS analysis. Peptide masses acquired from the MS/MS were compared to a *D. immitis* genome and the *Canis familiaris* genome (Nesvizhskii et al., 2003). The program Blast2Go was utilized to analyze proteins identified as being from *D.
D. immitis by mass-spectrometry (Conesa et al., 2005; Hewitson et al., 2008). 110 proteins were found in the D. immitis in vitro secretome. Of these, 52 were found to be unique to the D. immitis secretome. In addition, it was determined that the secreted proteins predominately fell into two major functional categories: catalytic activity and binding (Geary et al., 2012). It had been reported previously that the closely related filarial nematode B. malayi secretome contains 228 proteins, and the protein functions predicted for these proteins were similar to those proteins identified in D. immitis (Moreno et al., 2008). These findings are consistent with two other studies in which 80 B. malayi ES proteins (Hewitson et al., 2008) and 852 B. malayi ES proteins (Bennuru et al., 2009) were identified.

Identification of D. immitis in vivo Secretome

The in vivo D. immitis secretome has yet to be determined. It is plausible that many of the ES proteins previously detected in vitro will not be detectable in vivo due to the host’s immune response and the differences between the in vivo and in vitro environments. It is hypothesized that many of the ES proteins are sequestered in immune complexes by the host’s immune response. Sequestration of proteins in immune complexes occurs for several other infectious organisms, including Wuchereria bancrofti (Brunner et al., 2000; Dixit et al., 2007; Michaud et al., 2010). It would be highly advantageous to determine which proteins are present and detectable in vivo, as well as which proteins are most abundant. The antigens currently used for diagnostic purposes are
released primarily from mature adults. Identification of additional antigens that allow for the detection of immature parasites is essential for providing early treatment. This knowledge is important in the determination of treatment strategies as it will enable the development of new antigen based diagnostic tests for abundantly secreted proteins in filarial infections.

The antigen detection assays currently used do not employ well-described antigens. In addition, the correlation between the quantity of antigen found in serum and parasite burden in the host is unknown. It is difficult to determine if the overall sensitivity of the current tests is adequate for use as a basis for comparing treatment strategies (Ayong et al., 2005; Gass et al., 2012; Harnett et al., 1998; Weil et al., 1997). Therefore, an examination of the *D. immitis* ES proteins found *in vivo* to identify suitable antigens as potential targets for quantitative immunodiagnostic development is expedient.

**Filarial Nematode Excretory-Secretory Mechanisms**

A parasite’s ability to establish residency in a host depends on proteins secreted by specific mechanisms or pathways. Three pathways of protein secretion are known in metazoans. The first is known as the classical secretory protein transport pathway. This pathway is involved in the export of signal peptide proteins to the cell surface or to the extracellular space. Proteins transported via this pathway enter the endoplasmic reticulum (ER) by way of specific signal peptide receptors. These proteins are then incorporated into
vesicles, which can exit the ER and make their way to the Golgi apparatus where they can be processed and sorted before being sent to their final destination.

The remaining two pathways involved in protein secretion are identified as non-classical pathways. The first of these involves proteins that have signal peptides, which interact with the ER as in the classical pathway, but function in a Golgi-independent manner. The second non-classical pathway is typically utilized by certain cytoplasmic and nuclear proteins that lack ER signal peptides. These proteins exit cells in both a Golgi and ER independent manner (Davis et al., 1980; Nickel et al., 2009).

Parasitic nematodes employ specialized excretory and secretory systems, including secretory gland cells, the gut, uterine fluids, and shedding of surface components. In the model organism *Caenorhabditis elegans*, the excretory canal cells, duct cells, pore cells, and excretory gland cells are involved in the excretory-secretory system (Altun et al., 2012). Fluids within the excretory canal of the worm are collected by the excretory gland cells which then release the fluid outside the worm through the excretory and pore cells connected to the excretory gland cells via gap junctions (Figure 3). Both the duct cells and the pore cells surround the excretory duct and are also connected by gap junctions (Nelson et al., 1983; Nelson et al., 1984).
Interest in the study of exosomes and their role in protein transport has recently intensified. Inward budding of endosomal membranes results in the formation of multivesicular bodies (MVBs). When MVBs fuse with the plasma membrane, they are released into the extracellular space as exosomes (Li et al., 2006). Exosomes function to transfer material between cells, promoting intercellular communication, and to eliminate obsolete proteins during cell maturation. There is also evidence that exosomes play a role in modulating...
immune responses (Fevrier et al., 2004; Li et al., 2006; Skokos et al., 2001). In addition, exosome release is a general mechanism for protein and RNA secretion by Leishmania ssp. (Silverman et al., 2010), and the release of exosomes occurs in apical secretion pathways in C. elegans (Liegois et al., 2006). Although exosome protein transport is a relatively new area of investigation, the fact that exosomes are known to play a role in secretion mechanisms in various organisms indicates that this transport pathway cannot be overlooked when considering filarial nematode ES protein secretion.

Additional contributing factors to nematode ES protein secretion include the fluid release that accompanies the shedding of eggs by the female adult worm (Frontera et al., 2003), and the release of surface molecules from the nematode cuticle which contributes substantially to the overall ES proteins detected in vitro (Lightowlers et al., 1988; Maizels et al., 1982; Wang et al., 2013). Numerous studies indicate that the nematode cuticle is a site of active protein shedding; in addition, changes in the surface composition of the cuticle have been noted over the course of the life cycle in many nematode parasites including adult Trichinella spiralis (Philipp et al., 1981), Toxocara canis (Maizels et al., 1984), Nippostrongylus brasiliensis (Maizels et al., 1983), and both the adult and MF of B. malayi and Brugia pahangi (Maizels et al., 1985).
Sequestration of *D. immitis* Proteins

During the host immune response to infection, parasite ES proteins secreted *in vivo* are sequestered by the host in immune complexes. Antigen-antibody immune complexes are a result of the immune response and effectively bind proteins that are secreted by parasites (Simon *et al.*, 2007). Secreted proteins that become bound in immune complexes could prove useful as markers of infection for diagnostic purposes. Following antibody-mediated sequestration of antigen, the complexes can remain detectable in the blood for many years. Initially, the immune response produces several different classes of antibodies towards foreign proteins, primarily IgM and IgG. IgG is produced throughout the primary and secondary immune responses, and consequently would be most abundant in circulating blood (Raja *et al.*, 2006; Simon *et al.*, 2007). The presence of circulating immune complexes can cause a broad spectrum of proinflammatory effects, including induction of cytokine secretion and activation of the complement cascade. Immune complexes can also deposit in tissues and vessel walls, leading to inflammation and tissue damage (Senbagavalli *et al.*, 2011). Circulating immune complexes have been isolated and found to contain proteins originating from a variety of pathogens including *Borrelia burgdorferi* (Brunner *et al.*, 2000), *Mycobacterium tuberculosis* (Raja *et al.*, 2006), *Schistosoma mansoni* (Bout *et al.*, 1977), and *Plasmodium falciparum* (Leech *et al.*, 1984). Based on what is known regarding the host immune response to parasitic organisms, it is highly probable that the ES proteins secreted *in vivo* by *D. immitis* will be recognized by antibodies in infected dog serum. Since the *in
vitro secretome of *D. immitis* is now known, an attempt to isolate and purify immunogenic proteins bound in the immune complexes of dogs infected with heartworm is timely and has the potential to lead to new diagnostic targets and potential vaccine candidates (Geary *et al.*, 2012; Simon *et al.*, 2007).

**Hypothesis**

Knowing that a host will produce an immune response to filarial nematode parasites upon infection, we hypothesize that antibodies from infected dog serum will recognize immunogenic ES proteins secreted by *D. immitis in vivo*. The main focus of this project is to compare the *in vivo* secretome to the *in vitro* secretome; do parasites secrete the same proteins in both conditions? The *in vivo* proteins identified by this study are not expected to be fully representative of the *in vitro* secretome due to the numerous differences in parasite physiology when cultured versus parasite physiology *in situ* and the challenges of recovering antigens from a parasite in the protein rich serum compartment.

**Project Objectives**

**Contributions to the Scientific Community**

Finding more effective treatment strategies against filarial nematode pathogens such as *D. immitis* is a priority for both the scientific and medical communities. While the identification of *D. immitis* ES proteins secreted *in vivo*
will primarily provide information for the development of diagnostic assays that could be adapted for a variety of filarial nematode infections, this identification could also provide insight into vaccine candidates. Determining which proteins are secreted \textit{in vivo} will add to our knowledge of how these parasites modulate the host environment. For all of these reasons, this project is timely and will significantly contribute to the scientific knowledge of host-parasite interactions during \textit{D. immitis} infection.

\textbf{Objectives}

This project has two distinct objectives. The first is to validate whether the \textit{D. immitis} ES proteins found \textit{in vitro} are representative of the \textit{in vivo} secretome. This involves isolating \textit{D. immitis} ES proteins that are recognized by host antibodies produced in the immune response to the parasite. These proteins will be identified by tandem mass-spectrometry. The second objective is to determine which \textit{D. immitis} ES proteins detected \textit{in vivo} could make good diagnostic targets. This will involve a direct comparison of the proteins found \textit{in vivo} to those previously identified \textit{in vitro} to determine which proteins could aid in the development of diagnostics or potential vaccines. Proteins secreted at higher abundance are ideal candidates for the development of more sensitive assays as greater quantities of protein would be available for detection in host serum. While previous work has been done to identify individual proteins which are reactive to host immune sera, this is the first systematic approach attempted to identify
immunogenic proteins from *D. immitis* (*Frank et al., 1996; Frank et al., 1996; Gonzalez-Miguel et al., 2010; Oleaga et al., 2009*).
CHAPTER II

METHODS
Methods

Culturing of Adult *D. immitis*

Sixty-four mixed sex, adult *D. immitis* were collected from dogs that were test-positive for MF. These procedures were approved by the Animal Use Committee of St. Matthew’s University School of Veterinary Medicine (Grand Cayman, British West Indies). Parasites were placed directly into culture fluid in Petri dishes (1 parasite/4 mL RPMI 1640 medium) at 39°C. 20 mM HEPES, 200 IU/mL penicillin, 200 mM L-glutamine, 200 IU/mL streptomycin, 25 μg/mL amphotericin B (Gibco Invitrogen, Grand Island, NY), 1% w/v D-glucose and 1% w/v sodium bicarbonate, (pH 7.2) were added to the culture medium immediately prior to the onset of culture. Every 24 hr, unhealthy and immotile worms were removed and the healthy worms were placed in fresh medium. The culture medium was collected over 3 days. Protease inhibitors (Complete EASYpack Roche, Indianapolis, IN) were added to the collected medium each day for storage at 4°C (Geary *et al*., 2012).

Concentration of Culture Media

Media were centrifuged at 1000 x g for 5 min to pellet MF released during culture. The supernatant was passed through a 0.22 μm filter and frozen at −20°C for shipment to the Institute of Parasitology, McGill University. There, the combined volume was concentrated to 10 mL using an Amicon Ultra 3000
MWCO (Millipore, Billerica, MA). Proteins were precipitated using 20% trichloroacetic acid (TCA). Protein pellets were washed with acetone (−20° C) 3 times before being allowed to air dry (Hewitson et al., 2008; Moreno et al., 2008). Protein pellets were re-suspended in phosphate buffered saline (PBS) and were subsequently pooled.

**Purification of Canine Antibodies**

Pooled sera from heartworm infected dogs were passed through a spin column containing 1 mL protein-A resin (Thermo Scientific) to retain IgG antibodies. The column was equilibrated using PBS (pH 7.2). The serum sample was diluted 1:1 in 0.1 M sodium phosphate, 0.15 M sodium chloride (pH 7.2). 2 mL of this sample was added to the protein-A column which was subsequently placed in a 15 mL conical collection tube. The sample was incubated at room temperature with end-over-end shaking for 1 hr. The column was subsequently centrifuged for 1 min at 1000 x g. The column was then washed 5 times using 2 mL PBS and centrifuged for 1 min at 1000 x g each time.

100 μl of 1 M Tris (pH 8.5) was added to five 15 mL collection tubes and the column was placed in the first of these tubes. 1 mL of 0.1 M glycine (pH 2.7) was added to the column, and the column was centrifuged at 1000 x g for 1 min. This was repeated 5 times using a new collection tube with 1 M Tris each time. Absorbance at 280 nm was measured using a Nanodrop instrument to monitor
protein elution. Fractions containing purified antibodies were concentrated using an Amicon Ultra 3000 MWCO filter (Millipore, Billerica, MA).

**SDS-PAGE and Western Blotting of ES Proteins**

Parasite ES proteins previously collected were run on a SDS-PAGE gel (10 %) and were subsequently transferred to a nitrocellulose membrane for Western blotting. This membrane was probed with the purified dog antibodies to ensure that parasite antigens were recognized.

**Homogenization of Adult *D. immitis* Worms**

Adult female *D. immitis* and blood from heartworm-infected dogs were obtained from the Filariasis Resource Reagent Center (FR3) in Athens, Georgia, USA. Two adult females were placed in a sterile mortar containing liquid N$_2$ and ground into a fine powder using a pestle. The powder was re-suspended in PBS and passed through 20 and then 30 gauge needles to achieve full dispersal, as documented by examination under light microscopy.
Collection of MF from Blood and Homogenization

Infected dog blood was centrifuged at 2000 x g for 10 min. The supernatant was passed through a 25 mm membrane via syringe (5 μm pore size) (Millipore Isopore TMTP filter). Retained MF were washed 3 times with PBS. The filter membrane was placed inside a 15 mL conical tube and rinsed with PBS. The membrane was removed and the tube was centrifuged at 1000 x g to pellet MF. MF were homogenized via numerous syringe and needle passes using a 20 and then a 30 gauge needle. The whole MF extract was examined via compound light microscopy to ensure disruption and homogenization.

Crosslinking Canine IgG to Protein-A Columns

10 μg of the previously purified antibodies were added to each protein-A mini-column, which were subsequently placed in 1.5 ml Eppendorf tubes. Columns were incubated at room temperature with end-over-end shaking for 1 hr and then centrifuged for 1 min at 1000 x g. A solution of 2.5 mM disuccinimidyl suberate (DSS) in DMSO was added at 10 X molar excess to the protein-A resin in the columns (Thermo Scientific). The crosslinking reaction was allowed to proceed for 60 min at room temperature with mixing. Columns were then centrifuged at 1000 x g for 1 min. 50 μL of 0.1M glycine-HCl (pH 2.7) was added to each column, and they were centrifuged to remove non-cross-linked antibodies and stop the crosslinking reaction. This was repeated 3 more times. The
columns were then washed with 0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol (pH 7.4) three times.

**Protein-A Column Purification of ES Proteins**

Protein samples for *D. immitis* ES, adult female extract, and MF extract were individually diluted in 0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol (pH 7.4). 500 μL aliquots of each sample were added to columns with cross-linked canine IgG, which were then incubated with mixing for 2 hr. Columns were centrifuged at 1000 x g for 1 min and washed with 200 μL of the same solution and centrifuged at 1000 x g for 1 min (repeated 4 times). 10 μL of 0.1 M glycine-HCl (pH 2.7) was added to each column before centrifuging at 1000 x g for 1 min to elute proteins bound to IgG (Figure 4). To neutralize the pH, 5 μL of 1 M Tris (pH 9.5) was added to each collection tube before centrifugation.
Figure 4. Crosslinking canine antibodies to Protein-A column and immunogenic protein purification. Step 1. Canine antibodies are bound and crosslinked to Protein-A beads in an affinity column. Step 2. Protein sample is added to the column where immunogenic proteins are recognized by bound antibodies. Step 3. Unbound non-immunogenic proteins are washed away. Step 4. Immunogenic proteins are collected during elution.
**Washing and Trypsinization of ES Protein**

Proteins (10 µg) were washed 3 times on an Amicon 3 kDa filter with 50 mM ammonium bicarbonate buffer. After elution, the sample was vacuum-dried in a SpeedVac and kept at -20° C until trypsin digestion. Proteins were solubilized in 25 µl 50 mM ammonium bicarbonate-1% sodium deoxycholate and heated at 95° C for 5 min. Samples were reduced with DTT (0.2 mM) at 37° C for 30 min and alkylated iodoacetamide (0.9 mM) at 37° C for 20 min. Finally, sequence grade trypsin (Promega; 1 µg) was added and sample incubated overnight at 37° C. The trypsin reaction was stopped by addition of 3% acetonitrile-1% TFA-0.5% acetic acid. Peptides were concentrated on stage tip (C18) and vacuum dried before MS injection.

**Tandem Mass-Spectrometry**

Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass-spectrometry (ES MS/MS). The experiments were performed with an Agilent 1200 nano pump connected to a 5600 mass-spectrometer (AB Sciex, Framingham, MA, USA) equipped with a nanoelectrospray ion source. Peptide separation took place on a self-packed PicoFrit column (New Objective, Woburn, MA) packed with Jupiter (Phenomenex) 5u, 300A C18, 15 cm x 0.075 mm internal diameter. Peptides were eluted with a linear gradient from 2-50 %
solvent B (acetonitrile, 0.1 % formic acid) in 90 min, at 300 nL/min. Mass spectra were acquired using a data-dependent acquisition mode using Analyst software version 1.6. Each full scan mass spectrum (400 to 2000 m/z) was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion (30 sec exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

Analysis of Mass-Spectrometry Results

All MS/MS samples were searched against a FastA dataset of predicted tryptic peptides derived from a genome sequence of *D. immitis* (see: nematodes.org/downloads/959nematodegenomes/blast/db2/nDi.2.2.2.aug.proteins.fasta.gz) and the *C. familiaris* genome in the NCBI database, using Mascot (Matrix Science, London, UK; version 2.3.02). Mascot was set up to search the Uniref100-Fabales database (release 12-05) assuming the digestion enzyme = trypsin. Mascot was searched with a fragment ion mass tolerance of 0.10 Da and a parent ion tolerance of 0.10 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modification. Two missed cleavages were allowed.

Scaffold (version 4.0.1), Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications
were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003).
CHAPTER III

RESULTS
Results

Concentration and Complexity of Collected Proteins

Approximately 35 μg protein was recovered from the 2 adult female *D. immitis* worms used to generate the adult extract; approximately 5 μg were submitted for MS/MS analysis. SDS-PAGE with coomassie blue staining confirmed a complex mixture of proteins present in the adult extract (Figure 5.a). Approximately 25 μg of protein was collected from the combined 24, 48, and 72 hr adult *D. immitis* culture media. SDS-PAGE with silver staining confirmed a moderately complex mixture of proteins present in the sample (Figure 5.b). Approximately 7 μg ES protein was submitted for MS/MS. Approximately 400,000 MF were used to generate the MF extract sample; 15 μg protein were recovered and approximately 5 μg submitted for MS/MS.
Figure 5.a Coomassie stained 4-12% gradient polyacrylamide gel. Lane 1 is Benchmark Prestained ladder, lane 2 is *D. immitis* adult female proteins pre-Protein-A column and lane 3 is post-Protein-A column. Figure 5.b Silver stained 4-12% gradient polyacrylamide gel. Lane 1 contains ES proteins post-Protein-A column, lane 2 is Benchmark Prestained ladder.
Confirmation of Canine Antisera Recognition of Protein Samples

Parasite ES proteins previously collected were run on a SDS-PAGE gel (10 %) and were subsequently transferred to a nitrocellulose membrane for Western blotting. At the same time, the female extract was electrophoresed and transferred. This membrane was probed with the Protein-A affinity purified canine antibodies to ensure that parasite antigens were recognized. The antisera recognized several proteins in both the female extract and the ES protein samples (Figure 6). A complex mixture of proteins was identified by the antisera, and several strong bands were present. The numerous strong bands observed in the female extract and the ES indicate that there are proteins in the samples that are significantly immunogenic in the canine host and are recognized by the purified canine whole antibodies. With this knowledge, the isolation and purification of immunogenic proteins was made plausible.
Figure 6. Western blot probed with purified antibodies from heartworm-infected dogs. Lane 1 represents adult female proteins post-Protein-A column, lane 2 represents ES proteins post-Protein-A column.
Mass-Spectrometry Analysis of Immunogenic Proteins

MS/MS analysis of the immunogenic proteins recognized by heartworm-infected dog IgG antibodies identified 12 proteins in the adult female extract, 9 in the MF extract, and 6 in the ES sample (Table 1). Four proteins were identified in all three samples: glyceraldehyde-3-phosphate dehydrogenase, P22U, fructose-bisphosphate aldolase, and polyprotein antigen. Heat shock protein 60, heat shock protein 70 and a galectin were identified in the adult female and MF extracts but not in the ES. The pepsin inhibitor Dit33 was present in the adult female extract and ES samples, while an actin protein was shared between the MF extract and ES. 2,3-diphosphoglycerate-independent phosphoglycerate mutase was found only in the MF extract, and paramyosin, intermediate filament protein, P27, and thioredoxin peroxidase were seen exclusively in the adult female extract. The previous study (Geary et al., 2012) identified 5 of the proteins found in this study: actin, intermediate filament protein, heat shock protein 70, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase 1. These 5 proteins were not identified as abundantly released proteins in the secretome, indicating that immunogenicity is a separate variable from abundance.
<table>
<thead>
<tr>
<th><strong>Dirofilaria immitis</strong> Adult Female - Sequence Description</th>
<th><strong>Abundance Rank</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Paramyosin</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate filament protein</td>
<td>2</td>
</tr>
<tr>
<td>Pepsin inhibitor Dit33</td>
<td>3</td>
</tr>
<tr>
<td>Galectin</td>
<td>4</td>
</tr>
<tr>
<td>Glyceraldehyde 3 phosphate dehydrogenase</td>
<td>5</td>
</tr>
<tr>
<td>P27</td>
<td>6</td>
</tr>
<tr>
<td>Thioredoxin peroxidase</td>
<td>7</td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td>8</td>
</tr>
<tr>
<td>P22U</td>
<td>9</td>
</tr>
<tr>
<td>Cuticular antigen tandem repeats</td>
<td>10</td>
</tr>
<tr>
<td>Heat shock protein 60</td>
<td>11</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Dirofilaria immitis</strong> Microfilaria - Sequence Description</th>
<th><strong>Abundance Rank</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>1</td>
</tr>
<tr>
<td>P22U</td>
<td>2</td>
</tr>
<tr>
<td>Glyceraldehyde 3 phosphate dehydrogenase</td>
<td>3</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>4</td>
</tr>
<tr>
<td>2,3-diphosphoglycerate-independent phosphoglycerate mutase</td>
<td>5</td>
</tr>
<tr>
<td>Heat shock protein 60</td>
<td>6</td>
</tr>
<tr>
<td>Polyprotein antigen</td>
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</tr>
<tr>
<td>Heat shock protein 70</td>
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</tr>
<tr>
<td>Galectin</td>
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<table>
<thead>
<tr>
<th><strong>Dirofilaria immitis</strong> Mixed-Sex ES - Sequence Description</th>
<th><strong>Abundance Rank</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyprotein antigen</td>
<td>1</td>
</tr>
<tr>
<td>P22U</td>
<td>2</td>
</tr>
<tr>
<td>Pepsin inhibitor Dit33</td>
<td>3</td>
</tr>
<tr>
<td>Actin</td>
<td>4</td>
</tr>
<tr>
<td>Glyceraldehyde 3 phosphate dehydrogenase</td>
<td>5</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1. Immunogenic *Dirofilaria immitis* proteins identified by tandem mass-spectrometry (MS/MS). Proteins are shown in rank order of abundance.
Examination of Identified Proteins

Examination of the proteins from the female extract, the MF, and the ES indicated that 4 were shared between all three groups. Six were shared between the female extract and the MF, 4 were shared between the female extract and the ES, and 5 were shared between the ES and MF samples (Figure 7).

Figure 7. Venn diagram illustrating the number of unique and shared proteins identified in the female extract, microfilaria, and excreted secreted protein samples that were submitted for mass-spectrometry analysis.
The Blast2Go analysis tool was utilized to mine the gene ontology (GO) based data to shed light on the processes and functions in which the proteins identified in this study are putatively involved. The annotation was improved by utilizing the Annotation Expander (ANNEX) and by adding the GO terms related to functional domains from scanning the InterPro database (Al-Shahrour et al., 2004; Bluthgen et al., 2005). This analysis revealed that, among the female extract, MF, and ES protein subsets, catalytic activity and binding were the two major molecular function categories (Figure 8).

Figure 8. Distribution of Gene Ontology terms (Molecular Function - level 2) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.
Other molecular function categories include structural molecule activity, enzyme regulator activity, and antioxidant activity. At a higher level of ontology (Figure 9), most of the assigned molecular activity could be assigned to nucleotide binding, hydrolase activity, and cation binding.

![molecular_function Level 4](image)

Figure 9. Distribution of Gene Ontology terms (Molecular Function - level 4) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.

The most common biological function categories (Figure 10) were cellular process, developmental process, metabolic process, and multicellular organismal process. Other categories included growth, biological regulation, response to stimulus, reproduction, and others.
Figure 10. Distribution of Gene Ontology terms (Biological Process - level 2) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.

A higher level of ontology (Figure 11) shows that most of the cellular and metabolic processes are related to biological development. GO Terms such as multicellular organismal development, anatomical structure development, primary metabolic process, and regulation of biological process are among the most common at this level of ontology.
Figure 11. Distribution of Gene Ontology terms (Biological Process - level 3) for immunogenic proteins identified from *Dirofilaria immitis* female extract, MF, and ES protein samples.
CHAPTER IV

DISCUSSION
**Discussion**

**Previous Studies and Implications**

The filarial nematode *Dirofilaria immitis*, the canine heartworm, is a widespread and important veterinary pathogen and is a useful model for filarial parasites of humans. An analysis of proteins released from adult *D. immitis* worms in culture is available (Geary *et al.*, 2012). However, although many proteins have been individually isolated from infected canines (Frank *et al.*, 1996; Oleaga *et al.*, 2009), this is the first time a systematic approach has been attempted to identify the *in vivo* secretome of a filarial nematode. The proteins identified in our experiments via MS/MS analysis are unique in that we evaluated ES proteins separately from adult somatic proteins. A few previous studies have attempted to look at sets of proteins from *D. immitis*. These included proteins identified by either MS/MS or two-dimensional electrophoresis in adult worm somatic extracts that reacted against antisera from heartworm-infected dogs, cats, and humans (Gonzalez-Miguel *et al.*, 2010; Gonzalez-Miguel *et al.*, 2010; Oleaga *et al.*, 2009). These studies identified immunogenic proteins involved in up-regulation of Th2 anti-inflammatory responses, detoxification, plasminogen binding, parasite metabolism, and others. The identification of the most abundantly secreted parasite proteins present in the serum of infected hosts offers a rational approach to the development of new diagnostic assays that may be applicable across the Filarioidea.
Reviewing Goals of the Project

We sought to identify *D. immitis* ES proteins found *in vivo* to validate the recently published *in vitro* secretome (Geary et al., 2012), and to investigate the proteins identified as potential diagnostic reagents. These goals were both accomplished through MS/MS analysis and direct comparison to the *in vitro* secretome. Six, nine and twelve proteins were identified in the ES, adult female, and microfilaria samples, respectively. This identification of ES proteins of filarial nematodes *in vivo* may lead to future development of diagnostic reagents. The results and information gleaned from this work are both timely and an important requirement for the development of methods to determine level of infection in the host, especially for human filariae.

*D. immitis* as a Model

The diverse mechanisms utilized by *D. immitis* to alter the pathological consequences of the host are still not well understood. What is known is that the complex relationships between *D. immitis* and its host result in the long lasting survival of the parasite as a chronic infection (Simon et al., 2012). Many filarial nematodes are characterized by their location in the deeper tissues of the host where they are harder to study. *D. immitis* is the most important filarial nematode in veterinary medicine and is found, primarily, in the left ventricle and pulmonary arteries, where it is more easily accessible for study than other filarial species. *D.*
*immitis* and their animal hosts are useful as models for studying other filarial nematodes, especially when focusing on the *in vivo* environment. While several species of filarial nematodes can infect humans, many of these parasites do not have a facile animal model that can be utilized for research. One important example of this is the causative agent of river blindness, *Onchocerca volvulus*, which only infects humans (Allen *et al.*, 2008; Taylor, 1990). A model such as *D. immitis*, which will readily infect several species, can be an extremely useful tool for the study of biology of human infection with filarial nematodes and can aid researchers in the search for better drugs and diagnostic reagents (Lee *et al.*, 2010).

**Mimicking the *in vivo* Environment**

The information available regarding the protein composition of the *D. immitis* secretome is still limited when compared to what is known for *B. malayi* (Simon *et al.*, 2012). The first proteomic analysis of the secretome *in vitro* is a large step in the right direction to further understand which proteins *D. immitis* utilizes to modulate the host immune response (Geary *et al.*, 2012). However, the *in vitro* environment may be significantly different from the *in vivo* environment with regard to ES protein release. For this reason, it was decided to utilize host immune serum to detect parasite proteins that the immune response has recognized during active infection. This should give a definite indication of which immunodominant proteins are released by the parasite *in vivo*, information
which can then be extrapolated to determine a subset of the \textit{in vivo} secretome, namely, the most immunogenic ES proteins. This would appear to be the most straightforward method for isolating at least some of the \textit{D. immitis} proteins released \textit{in vivo}.

**Protein Isolation and Purification**

Adult \textit{D. immitis} obtained from infected dogs were maintained in culture for 72 hr with daily changes of media. Proteins were concentrated from spent media by standard methods and passed through Protein-A columns containing purified IgG antibodies from heartworm-infected dogs. The fact that numerous proteins were identified by these methods is encouraging, as a review of the literature has shown that a systematic approach such as this has not been attempted before to detect proteins from the secretome of any filarial nematode.

**MS/MS Analysis and Proteins Identified**

It was decided to analyze the collected immunogenic proteins by an in-solution tryptic digestion followed by a 2 hr separation with reverse-phase (RP) nanoLC and analysis by ES MS/MS. The two hr separation was chosen because it is twice as long as a normal run, and would provide better separation of peptide sequences leading to a more precise analysis of the proteins in each sample. The longer separation time also helped to unmask lower abundance peptides.
The mass-spectrometry was done using a TripleTOF 5600 mass-spectrometer (AB Sciex, Framingham, MA, USA). This is a hybrid quadrupole time-of-flight (QqTOF). Since the TripleTOF is coupled to a capillary HPLC for peptide separation via a nanospray ionization source, a resolution of more than 30,000 Full Width at Half Maximum (FWHM) is possible. The high speed and resolution capabilities of the TripleTOF makes it an excellent choice for analyzing complex protein mixtures (Jones et al., 2013).

We elected to identify parasite proteins that elicited an antibody response. Of the 12 proteins identified by this method in adult female extract, 4 were also detected in the *D. immitis* secretome. Of the 9 proteins identified in the MF extract, 4 were previously identified in the secretome. Of the 6 proteins identified in the ES fraction, 3 were also found to be previously secreted in culture (actin, glyceraldehyde 3 phosphate dehydrogenase, and fructose-bisphosphate aldolase; (Geary et al., 2012). These data support the general reliability of the culture system used to identify ES proteins, but also suggest that hosts react to proteins released from dead or dying MF or adult parasites.

Polyprotein antigen, paramyosin, heat shock protein 70, and intermediate filament protein have all been localized to the surface of the parasite and have attracted attention as potential diagnostic and vaccine candidates for *D. immitis* and related filarial parasites (Cho-Ngwa et al., 2011; Poole et al., 1996; Ravi et al., 2004; Zhang et al., 2011). Although the P22U protein was not reported in the *D. immitis* secretome (Geary et al., 2012), it has been described as an ES protein in other studies and is characterized as a plasminogen-binding protein (Gonzalez-Miguel et al., 2013). The P27 protein belongs to the small heat shock
protein family and is associated with molting (Lillibridge et al., 1996). The pepsin inhibitor Dit33 has also been shown to be an ES protein (Willenbucher et al., 1993). The metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase, fructose bisphosphate aldolase, and 2,3-diphosphoglycerate-independent phosphoglycerate mutase have been identified as detectable antigens during *D. immitis* infection and are involved in glycolytic pathways (Gonzalez-Miguel et al., 2013; Li et al., 2011).

The findings of this study are strengthened by the fact that the majority of the proteins identified are significantly immunogenic in *D. immitis*, or other filarial species. Dit33 is recognized by infected host immune serum in *D. immitis*, *B. malayi*, and *O. volvulus* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010; Hong et al., 1996; Oleaga et al., 2009). The galectin we identified was immunogenic and reactive to infected host serum in *D. immitis*, *B. malayi*, and *Haemonchus contortus* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010; Oleaga et al., 2009). Glyceraldehyde-3-phosphate dehydrogenase was identified as immunogenic in *B. malayi* and *W. bancrofti* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010; Oleaga et al., 2009). Fructose-bisphosphate aldolase was immunogenic in *B. malayi* and *O. volvulus* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010; McCarthy et al., 2002; Oleaga et al., 2009). Actin is immunogenic in *O. volvulus* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010; Oleaga et al., 2009). HSP70 has been identified as an immunogenic protein in *W. bancrofti*, *B malayi*, and *O. volvulus* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010; Oleaga et al., 2009; Rothstein et al., 1989). Paramyosin promotes a strong immune response during infection with *B.*
*malayi* (Langy et al., 1998). Host immune sera recognizes Intermediate filament protein in *O. volvulus* infection (Zhang et al., 1995). 2,3-diphosphoglycerate-independent phosphoglycerate mutase from *B. pahangi* was immunogenic in cats (Au et al., 1982). The P27 protein is also immunogenic in *D. immitis* (Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010). *D. immitis* thioredoxin peroxidase and P22U react to host immune serum, identifying them as immunogenic (Frank et al., 1996; Gonzalez-Miguel et al., 2010; Gonzalez-Miguel et al., 2010). Finally, the polyprotein antigen is immunoreactive in *D. immitis* (Culpepper et al., 1992), and cuticular antigen tandem repeats homologs are recognized during the host immune response to *B. malayi* (Kennedy, 2000; Yazdanbakhsh et al., 1995).

**Potential Issues Encountered**

The work done to isolate immunogenic *D. immitis* proteins faced several challenges. The relatively low recovery of parasite proteins achieved with this protocol is unsurprising, given the complexity of the process. Low abundance and mildly immunogenic proteins would be difficult to capture for MS/MS identification. Nonetheless, the proteins detected offer insights into the host-parasite interface.

The possibility that non-specific binding of non-immunogenic parasite protein to the purified host antibodies would occur was a substantial concern. However, the fact that essentially all of the proteins identified by MS/MS have
been shown to be immunogenic in *D. immitis* or other parasite species validates our approach and indicates that non-specific binding is not a significant drawback to this method.

Another concern that arose during the course of this research was that some immunogenic proteins would be masked during MS/MS by much more highly abundant proteins. This potential obstacle was circumvented by having each sample run for 2 hr instead of the typical 1 hr time. Doing this is expected to increase sensitivity to enable proteins of low abundance to be identified.

**Secretome Comparison and Identification of Diagnostic Candidates**

When comparing the *in vitro* *D. immitis* secretome to the immunogenic proteins identified here, which represent a subset of the *in vivo* secretome, some crossover is observed. Of the 12 proteins identified in adult female extract, 4 were also detected in the *in vitro* secretome (intermediate filament protein, glyceraldehyde 3 phosphate dehydrogenase, fructose-bisphosphate aldolase, and heat shock protein 70). Of the 9 proteins identified in the MF extract, 4 were previously identified *in vitro* (actin, glyceraldehyde 3 phosphate dehydrogenase, fructose-bisphosphate aldolase, and heat shock protein 70). Of the 6 proteins identified in the ES, 3 were also found to be previously secreted in culture (actin, glyceraldehyde 3 phosphate dehydrogenase, and fructose-bisphosphate aldolase). These data support the general reliability of the culture system used to identify ES proteins, but also suggest that hosts react to proteins released from
dead or dying MF or adult parasites and that not all proteins released in culture are immunogenic or released in high abundance in vivo.

All of the proteins found in this study could potentially be useful for identifying vaccine candidates depending on whether immunogenic proteins are selected or excluded. However, the immunogenic proteins identified here already have host antibodies produced towards them during the immune response and are unlikely to be useful vaccine candidates. When a host is exposed to a parasite protein which is immunogenic, antibodies will be developed which are clearly not protective. Several of these proteins could also prove useful as potential diagnostic reagents. In particular, it would be interesting to see whether proteins such as glyceraldehyde 3 phosphate dehydrogenase, or heat shock protein 70 could be used in the development of quantitative assays like a sandwich ELISA, since their homology is highly conserved between the parasite and the host. However, it would require additional investigation and laboratory work to determine which of these proteins might prove most useful for such an assay.

*D. immitis* infection, known as dirofilariasis, is an increasing problem on a global scale. New diagnostic techniques and treatments are necessary to address this problem, especially since resistance to many of the current treatments, including the macrocyclic lactones, has been recognized (Bourguinat et al., 2011; Geary et al., 2011). The utilization of the novel proteins identified in this study may provide new insight into effective ways to develop these tools.
**Future Directions**

Based on the results of our proteomic analysis, and the fact that we have determined that immunogenicity and abundance are separate variables, deciding which proteins would make good diagnostic targets is slightly more complex than it otherwise might have been had the immunogenic proteins also been among the most abundantly secreted *in vitro*. However, 5 of the proteins found in this study were also identified in the initial secretome study (Geary et al., 2012): actin, intermediate filament protein, heat shock protein 70, glyceraldehyde-3-phosphate dehydrogenase, and fructose-bisphosphate aldolase 1. Even though these 5 proteins were not previously identified as abundantly released proteins in the secretome, they are both immunogenic and released as ES products and, therefore, it would be worthwhile to do further research to analyze them as candidate proteins. It would be expedient to check the alignments between the host proteins and parasite homologs to see how similar they are, as some of the proteins, such as actin, are highly conserved. Based on this, development of quantitative, parasite-specific, assays to assess the level of infection for *D. immitis* which, in turn, could be extrapolated and used for other closely related filarial nematodes like *O. volvulus* and *W. bancrofti* would be worthwhile and highly advantageous. Utilizing the new knowledge of *D. immitis* immunogenic proteins from this study, it would be interesting to see whether there are highly secreted proteins in the secretome that are not very immunogenic in order to identify better candidates for development of vaccines. This would require an in-
depth analysis to determine similarity levels of the host and parasite homologs and, potentially, animal trials.

**Conclusion**

The identification of excreted-secreted proteins of parasitic filarial nematodes for use as diagnostic reagents is important for the development of more sophisticated methods to determine level of infection in the host and to identify individuals that require treatment. The investigation undertaken and detailed in this M.Sc. thesis sought to identify *D. immitis* ES proteins found *in vivo* as potential diagnostic targets.

*D. immitis* infection is a key veterinary concern that has a nearly global distribution. Unfortunately, there is a lack of effective quantitative tests to determine worm burden for many filarial parasite infections, including infection by *D. immitis*, thus making this investigation both expedient and timely. New diagnostic targets may be identified from the research presented here not only for infections by *D. immitis*, but also for infections with closely related filarial nematodes such as *O. volvulus*, which is a significant problem in human medicine.

This study validates the techniques utilized to isolate immunogenic *D. immitis* proteins. The culturing of adult parasites to collect ES proteins and the isolation of immunogenic proteins using antibodies obtained from heartworm-infected dogs was successfully accomplished. The antibodies recognized proteins in both the mixed-sex *D. immitis* ES proteins and adult female extract as
demonstrated by Western blotting. Tandem mass-spectrometry detected 12 immunogenic adult female proteins, 9 immunogenic microfilarial proteins, and 6 immunogenic ES proteins. Several of the proteins seen in this work were not previously identified as abundantly released proteins in the in vitro secretome. This would seem to indicate that immunogenicity is a separate variable from abundance. There was a great deal of overlap between the proteins in the female extract, MF, and ES proteins. Catalytic activity and binding were the two major molecular function categories exhibited by the proteins identified.

The information obtained from this work represents a considerable extension of the current knowledge on parasite proteins released into the host environment. The insights that this research grants will facilitate future studies on the proteins involved in the host-parasite interaction and on the development of diagnostic tools and potential vaccine candidates.
Bibliography


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