IMMUNE BASED DETECTION OF *ANGIOSTRONGYLUS VASORUM* INFECTION IN DOGS AND MOLECULAR CHARACTERIZATION OF THE ANTIGEN

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ABSTRACT

Angiostrongylus vasorum (French heartworm) is a metastrongylid nematode which infects the pulmonary artery and right ventricle of wild and domestic canids and can lead to fatal cardiopulmonary disease in infected dogs. In North America, there is an endemic focus of infection in Newfoundland, Canada. Diagnosis is made by detection of first-stage larvae by Baermann fecal examination, however, fecal diagnosis can lack sensitivity due to intermittent shedding of larvae. In this study we developed serological tests to detect French Heartworm similar to those used for the diagnosis of North American Heartworm (*Dirofilaria immitis*) infection. An initial attempt to use an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to the parasite was confounded by significant cross-reactivity to antigens of *Crenosoma vulpis*, which is a metastrongylid nematode that is also common in Atlantic Canada. We therefore developed a sandwich ELISA to detect circulating antigens of *A. vasorum* using rabbit polyclonal antiserum prepared against whole-worm adult antigen. A test positive cut-off value and test sensitivity and specificity were determined by two-graph receiver operating characteristic curve (ROC) analysis using sera from 24 Baermann positive *A. vasorum* dogs from Newfoundland and sera from 52 Baermann negative *A. vasorum* dogs from non endemic areas. An optical density of 0.19 was used as the positive cut-off and the test specificity was 100% with a sensitivity of 92%. Furthermore, sera from 30 *Crenosoma vulpis* positive dogs were tested and found to be negative on the sandwich ELISA. A survey of dogs from Newfoundland with signs of cardiopulmonary disease compared Baermann fecal results with the sandwich ELISA and indicated that fecal testing may have missed almost half of the infections. To standardize the test,

increase sensitivity, and develop a dependable source of diagnostic antigen(s), this study characterized some immunoreactive antigen(s) detected in the sandwich ELISA test. We developed a cDNA library from adult *A. vasorum* and after screening with rabbit anti-*A. vasorum* serum we identified 8 immunoreactive clones. Sequence analysis showed these clones to contain sequences homologous to 3 proteins, which shared homology to similar proteins in other parasites: vitellogenin, tropomyosin and heat shock protein 70 (Hsp70).

For production of specific monoclonal or polyclonal antibodies to be used in the sandwich ELISA test, it is necessary to produce a pure protein from the *A. vasorum* cDNA library. In the current study the most commonly occurring sequence encoded for a vitellogenin protein was chosen for expression and was inserted into a pGEX plasmid that was used to transform *E. coli* strain BL21. This vector incorporated glutathione S-transferase (GST) at the N-terminus and vitellogenin was synthesized as a fusion protein with GST. Western blotting with rabbit anti-*A. vasorum* polyclonal antiserum was able to identify the recombinant protein. This fusion protein had a molecular mass of 77 kDa which represented the combined mass of the GST (26 kDa) and the vitellogenin protein (51 kDa). The fusion protein was purified using GST affinity resin. Several modifications to the protocol for eluting the protein of interest from an affinity column had to be made including adjustments to pH, reduced glutathione buffer, salt concentration and Triton X-100 concentration.

In conclusion, a sandwich ELISA was developed and found to be more sensitive than the current Baermann test. Specific gene fragments from *A. vasorum* antigens were identified and sequenced. The most common sequence encoding for vitellogenin

protein was purified. The generation of specific proteins should result in the development of antiserum that will enable higher test sensitivity than that obtained using crude *A. vasorum* somatic antigen. Furthermore, this will potentially increase sensitivity of the sandwich ELISA test and allow dependable production of reagents.

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DEDICATION

I would to dedicate this thesis with all my love to my wonderful and amazing family but foremost to my parents Polina and Oleg Verzberger, my brother Igal, my in-laws, Shela and Yosef Epshtein, my husband Ariel who is my strength and inspiration and to my puppy Aesop who was very patient with me while I was writing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
AVC	Atlantic Veterinary College
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees celsius
cDNA	Complementary DNA
CI	Confidence interval
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Diothiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory/secretory
GST	Glutathione-S-transferase
Hsp70	Heat shock protein 70
Ig	Immunoglobulin

IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobyte
kDa	kilo Dalton
L1	First-stage larva
L3	Third-stage larva
L5	Fifth-stage larva
LB	Luria Bertani
LPG	Larvae per gram
MCS	Multiple cloning site
MOPS	Morpholinopropanesulphonic acid
mRNA	messenger RNA
NB	New Brunswick
NS	Nova Scotia
NZY	NZ amine (casein hydrolysate)
OD	Optical density
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% Tween-20
PCR	Polymerase chain reaction
PEI	Prince Edward Island
PI	Post-infection
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

RNase	Ribonuclease
ROC curve	Receiver operating characteristic curve
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STE	Sodium, Tris, EDTA
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TTBS	Tris buffered saline with 0.05% Tween 20
UK	United Kingdom

CHAPTER 1: GENERAL INTRODUCTION

1.1 HISTORY

Angiostrongylus vasorum (French heartworm) is a metastrongylid nematode which infects the pulmonary artery and right ventricle of wild and domestic canids. Foxes and dogs become infected by ingesting snails or slugs containing third-stage larvae. Clinical signs are not usually noticed until the chronic stage which may occur months or years after the initial infection. The effects upon the host range from subclinical to fatal infections (Bolt *et al.*, 1994). Wild foxes serve as infection reservoirs for domestic dogs (Bolt *et al.*, 1992). The parasite was first recovered from a dog during necropsy in 1853 in Toulouse, France, by Serres.

Gescheidt, a German ophthalmologist, reported in 1833 a canine ocular helminthosis involving an unknown nematode which appears to have been *A. vasorum.* Baillet was the first to describe *A. vasorum* in 1866, naming it *Strongylus vasorum* (Bolt *et al.*, 1994). Costa *et al.* (2003) recently redescribed *A. vasorum* and the following history is taken from that report. In 1905, Kamensky proposed the genus *Angiostrongylus*, based on his examination of *Strongylus vasorum* Baillet, 1866 and another similar protostrongylid parasite of the pulmonary artery and right heart of dogs, *Ematozoa filarial cardiaca* Bossi 1871. He renamed these parasites as *Angiostrongylus vasorum* and *Angiostrongylus cardiacus* respectively. In 1909, Railliet and Henry making no reference to Kamensky, proposed the genus *Haemostrongylus* for the species *S. vasorum.* Leiper in 1926 noted that *Angiostrongylus* had priority and *Haemostrongylus* was reduced to a synonym.

Dougherty (1946) recognized six species in the genus *Angiostrongylus*: *A. cantonensis*, *A. gubernaculatus*, *A. ondatrae*, *A. raillieti*, *A. tateronae* and *A. vasorum*. In 1951, to accommodate two species *A. cantonensis* and *A. gubernaculatus*, Schulz created two new genera, *Rattostrongylus* and *Angiocaulus* (Schulz, 1951). Skrjabin (1952) placed the *Angiostrongylus* species classified by Dougherty in four genera: *Angiostrongylus* (*A. raillieti*, *A. tateronae*, *A. ten* and *A. vasorum*), *Rattostrongylus* (*A. cantonensis*), *Angiocaulus* (*A. gubernaculatus*) and *Rodentocaulus* (*R. ondatrae*).

A systematic revision of the genus *Angiostrongylus* by Drozdz (1970), placed the species in two genera: *Angiostrongylus* (with two subgenera, *Angiostrongylus* and *Parastrongylus*) and *Stefankostrongylus* (species included: *S. blarini, S. michigaensis* and *S. soricis*). Grisi (1971) in a comparative study of *H. raillieti* and *A. vasorum* concluded that both species have a gubernaculum and proposed that they should be classified as *Angiocaulus raillieti.*

Costa *et al.* (2003) listed the following synonyms for *Angiostrongylus vasorum*: *Strongylus vasorum*, *Ematozoa filarial cardiaca*, *Haemonchus vasorum*, *Haemostrongylus vasorum*, *Haemostrongylus raillieti*, *Angiostrongylus raillieti*, *Angiocaulus raillieti*. Based on the high degree of similarity, Costa *et al.* (2003) reduced *Angiocaulus* to a synonym of *Angiostrongylus vasorum* and recognized 12 species in the genus of *Angiostrongylus* (Table 1.1).

1.2 TAXONOMY OF FAMILY ANGIOSTRONGYLIDAE

Angiostrongylus vasorum (French heartworm) belongs to the phylum Nemathelminthes, class Nematoda, order Strongylida, superfamily Metastrongyloidea and family Angiostrongylidae. The superfamily Metastrongyloidea is a group of bursate nematodes (Strongylida), commonly called lungworms because as adults they inhabit, with a few exceptions, the respiratory tracts of carnivores, ungulates, primates and rodents. Others occur in the vascular system or the nervous system (Urquhart *et al.*, 1996). They are found throughout the world and are important parasites of ruminants, pigs, dogs and cats and various species of wildlife. Metastrongylids do not occur in cattle and horses, their place as lungworms being occupied by *Dictyocaulus* species (Urquhart *et al.*, 1996).

Most metastrongylids include intermediate hosts in their life cycles, usually terrestrial gastropods, except the lungworm of swine, which uses the earthworm as an intermediate host. There are several unusual exceptions to this pattern of transmission. For example, in two species in dogs, *Oslerus osleri* and *Filaroides hirthi*, the life cycles are direct and, unusual in the nematodes, first-stage larvae are directly infective to their definitive hosts without any of the usual development to the third-stage larva (Urquhart *et al.*, 1996). The parasitic phases of the life cycles of these lungworms always include migration. Since transmission usually occurs by ingestion of intermediate hosts and following release by digestion in the gastrointestinal tract, the nematodes must migrate to reach their predilection sites in the respiratory system of the definitive host.

Angiostrongylus vasorum belongs to the family Angiostrongylidae. Species in this family have bursae that are well developed or reduced (but typical rays are usually

clearly defined) and the females have a vulva near the anus and are oviparous (Anderson, 1978). There are several species in the genus *Angiostrongylus*, the host, site of habitant and geographic distribution are summarized in Table 1.1 (Anderson, 1978; Costa *et al.*, 2003; Dougherty, 1946).

Table 1.1: The different species in the genus *Angiostrongylus*

Species of *Angiostrongylus*	Definitive Hosts	Organ	Geographic Distribution
A. blarini	Northern short-tailed shrew (*Blarina brevicauda*)	Lungs	North America
A. cantonensis	Rats, humans (*Rattus spp., Homo sapiens*)	Lungs and central nervous system	Hawaii, South Pacific, Asia, Australia
A. chabaudi	Wild cat (*Felis silvestris*)	Lungs	Europe
A. gubernaculatus	American badger, Island fox (*Taxidea taxus*, *Urocyon littoralis*)	Heart and pulmonary arteries	North America
A. macherrasae	Rats (*Rattus spp.*)	Pulmonary arteries and right ventricle of the heart	Australia
A. michiganensis	Masked shrew (*Sorex cinereus*)	Bronchioles	North America
A. sandarsae	Rats (*Rattus spp.*)	Pulmonary artery	Africa
A. schmidti	Marsh Rice Rat (*Oryzomys palustris*)	Lungs	North America
A. sciuri	Red squirrel (*Sciurus vulgaris*)	Lungs	Europe
A. soricis	Pygmy shrew (*Sorex minutus*)	Lungs	Europe
A. tateronae	Mice (*Muss pp.*)	Lungs	Africa, Europe
A. vasorum	Wild and domestic canids	Pulmonary arteries and right ventricle of the heart	Europe, Asia, South America, North America

1.3 MORPHOLOGY OF *ANGIOSTRONGYLUS VASORUM*

1.3.1 Adult nematodes

Angiostrongylus vasorum are small, slender and pinkish in color. Females are ~15-21 mm long and males are ~14-16 mm long. Females have a "barber pole" appearance due to the red intestine intertwined with the white reproductive tract. The ovaries contain numerous undifferentiated eggs. Males are bursate with the bursa being suspended from seven pairs of papillae. The spicules are ~ 400-500 µm long with the right being slightly shorter than the left. The gubernaculum is small and difficult to see (Guilhon and Cens, 1973).

1.3.2 First-stage larvae (L1)

The first-stage larvae are often very active and range in length from 310 to 399 µm long. The anterior portion is characterized by a cephalic button and the tip of the tail has a characteristic (sinus) wave appearance and a small dorsal spine (Ash, 1970).

1.3.3 Third-stage larvae (L3)

The third-stage larvae inside the intermediate host (the gastropods), are liberated from the gastropods tissue within the definitive host. Similar to first-stage larvae, they are translucent and mobile. Their size ranges ~ 508-610 µm long. The tip of the tail of the L3 is digiform with faint transverse cuticular striations. The esophagus is ~1/3 of the body length of the larvae. The genital primodium is in the distal third of the larva (Ash, 1970)

1.4 HOST RANGE AND GEOGRAPHIC DISTRIBUTION

1.4.1 Host range

The natural definitive hosts of *A. vasorum* are various species of wild foxes. Natural infections have been reported in red fox (*Vulpes vulpes*), African desert fox (*Fennecus zerda*), crab-eating zorros (*Cerdocyon thous*), hoary fox (*Dusicyon vetulus*), coyote (*Canis latrans*), wolf (*Canis lupus*) and domestic dogs (Bolt *et al.*, 1994; Bourque *et al.*, 2005; Lima *et al.*, 1994; Rosen *et al.*, 1970; Segovia *et al.*, 2001).

Patent experimental infections have been established in a jackal (*Canis aureus*) and Nile rats (*Arvicanthis niloticus*) (Bolt *et al.*, 1994). In experimental infections of cats (*Felis catus*), *A. vasorum* reached maturity but neither eggs nor larvae were produced (Bolt *et al.*, 1994; Guilhon and Cens 1970).

Kamenov *et al.* (1999) reported natural infection in two cats in Bulgaria with signs of chronic cough and metastrongylid first-stage larvae were recovered by Baermann examination. The larvae were ~ 430 µm long and had a kinked tail and dorsal spine. However, the length of the larvae cited and the tail morphology illustrated in the figures are inconsistent with *A. vasorum*, and larvae may be *Aelurostrongylus abstrusus*, the cat lungworm.

1.4.2 Geographic distribution

Angiostrongylus vasorum infection occurs in various parts of Europe (France, England, Denmark, Germany, Spain, Ireland, Italy, Hungary, Finland and Switzerland), Turkey, Africa (Uganda), countries of the former USSR, South America (Brazil, Colombia) and in North America (Newfoundland, Canada) (Bolt *et al.*, 1994;

Bwangamoi, 1974; Cobb and Fisher 1990; Davidson *et al.*, 2006; Dodd, 1973; Hayes and Rowlands, 2004; Jacobs and Prole, 1976; Lima *et al.*, 1994; Manas *et al.*, 2003; Poli *et al.*, 1983; Rosen *et al.*, 1970; Simpson and Neal, 1982; Smith and Threfall, 1973; Sreter *et al.*, 2003; Willingham *et al.*, 1996).

In Newfoundland, *A. vasorum* is prevalent in the southeastern portion including the Avalon and Burin Peninsulas, and along the eastern coast from Hamilton Sound to Bonavista Peninsula (Jeffery *et al.*, 2004).

1.5 NATURAL DEFINITIVE HOSTS

1.5.1 Wild canids

Foxes are considered the main natural definitive hosts for *A. vasorum* in the various endemic regions (Conboy, 2004). The red fox serves as a natural reservoir of infection in Europe and North America. Poli *et al.* (1983) reported an infection prevalence of 23% (43/180) in red foxes collected in the years 1981-1983 from various areas of Tuscany, Italy. Adult nematodes were found in the right ventricle and in the pulmonary artery. The number of parasites per infected fox ranged from 3-46 (Mean=18.8, SD=11.6). Poli *et al.* (1991) reported increased prevalence of *A. vasorum* infection in the red fox population in Tuscany, roughly doubling in animals surveyed from 1984-1988 compared to those from 1981-1983.

Baermann fecal surveys of red fox Denmark collected from the region north of Copenhagen indicated that 36%-80% were infected with *A. vasorum* (Bolt *et al.*, 1992; Willingham *et al.*, 1996). In a necropsy survey of red fox collected throughout Denmark, most of the *A. vasorum* infected animals occurred in the Northern Zealand

region where prevalence of 49% (225/463) was observed (Saeed *et al.*, 2006). Higher prevalence was found in adult animals (52%) compared to those 6-12 months of age (45%) or younger (32%). Although age estimates were imprecise, the youngest animal in which infection was observed was reported to be 1 month old.

Angiostrongylus vasorum was found in 42% (5/12) of the foxes in Cornwall, UK. Two foxes that had moderately heavy infection also had severe lesions of sarcoptic mange (Simpson, 1996). *Angiostrongylus vasorum* was not found in a survey of 169 red foxes in Norway but has been reported at low prevalence (2%) in Finland (Davidson *et al.*, 2006). Sreter *et al.* (2003) reported a prevalence of *A. vasorum* infection of 5% (5/100) in a necropsy survey of red fox in Hungary.

Angiostrongylus vasorum was first reported in 2 red foxes in Newfoundland by Smith and Threlfall (1973). A necropsy survey conducted in Newfoundland by Jeffery *et al.* (2004) examined 366 foxes collected through two separate trapping seasons from 2000 to 2001; 56% were infected with *A. vasorum*. There was an apparent increase in prevalence and spread in geographic distribution of the endemic region between the 2000 and 2001 trapping seasons. In 2000, 51% of the foxes were infected and *A. vasorum* was restricted to the Avalon Peninsula and North Coast. In 2001, 76% were infected and *A. vasorum* was also found in the North East Coast and on the South Coast and Burin Peninsula. Infection intensities ranged from 1-379 (Mean=72) adult nematodes; the majority (88%) were found in the pulmonary arteries and the remainder in the right ventricle. Ninety-five percent of the infected foxes had nematodes in the pulmonary arteries and 73% of the foxes had worms in both the pulmonary artery and right ventricle. Pulmonary arteries supplying all of the lung lobes were affected;

however more nematodes were recovered from the arteries of the caudal than craneal lung lobes. Prevalence did not change with age and there did not appear to be any effect of infection intensity on body condition scores.

In the southern part of the State of Minas Gerais, Brazil, a necropsy survey was conducted on 8 hoary foxes (*Dusicyon vetulus*) in 1993; 50% (4/8) were infected with *A. vasorum* (Lima *et al.*, 1994). In northwestern Spain, a necropsy survey of 47 wolves was conducted from 1993-1999, and *A. vasorum* was detected in 2.1% (1/47) of the animals (Segovia *et al.*, 2001). Necropsies were performed on 85 Eurasian badgers (*Meles meles*) from four different areas of Spain. *Angiostrongylus vasorum* was only found in the coastal region of the Mediterranean where it was recovered from 6.4% (3/47) of the badgers examined (Torres *et al.*, 2001). In Newfoundland, a female coyote was found on the road apparently dead by vehicle-related trauma. A fecal sample was collected and examined by Baermann technique, 349.4 first-stage larvae per gram were found. Adult nematodes (4) were also recovered from the pulmonary arteries (Bourque *et al.*, 2005).

1.5.2 Domestic dogs

Relatively few surveys have been conducted in the various endemic regions to establish prevalence of *A. vasorum* infection in dogs. Bwangamoi (1974) found 21% (26/123) of the dogs in Uganda were infected with *A. vasorum* at necropsy. *Angiostrongylus vasorum* was first diagnosed in the United Kingdom (UK) in 1975 in a Baermann fecal examination survey in which *A. vasorum* larvae were detected in 1/276 (0.4%) racing greyhounds (Jacobs and Prole, 1976). The infected dog had been

imported from Ireland. Martin *et al.* (1992) found an *A. vasorum*-infection prevalence of 4% (8/197) in a Baermann fecal survey of dogs in the Cornwall area of England. Seven of the 8 infected dogs were located within a 6-mile radius in the Redruth area of Cornwall. Discrete, small endemic foci of *A. vasorum* infection occur within the area of geographic distribution for the parasite in France (Bolt *et al.*, 1994).

A Baermann fecal survey of dogs suffering clinical signs consistent with cardiopulmonary disease conducted in Newfoundland, Canada, from 2000-2001 found that 24% (16/67) of the animals were infected with *A. vasorum* (Conboy, 2004).

1.6 LIFE CYCLE OF *ANGIOSTRONGYLUS VASORUM*

The life cycle of *A. vasorum* is indirect with gastropods serving as intermediate hosts. A wide range of mollusks including slugs (*Arion ater, A. hortensis. A. lusitanicus, Deroceras reticulatum, Limax flavus*), terrestrial snails (*Achatina fulica, Arianta arbustorum, Bradybaena similaris, Cepaea nemoralis*) and aquatic snails (*Biomphalaria glabrata, B. pfeifferi, Physa* sp.) have been experimentally infected with *A. vasorum* (Guilhon and Afghahi, 1969; Prestwood *et al.*, 1981; Rosen *et al.*, 1970).

The common frog (*Rana temporaria*) can act as a paratenic and as an intermediate host to *A. vasorum* (Bolt *et al.*, 1993). Frogs ingesting snails containing third-stage larvae or frogs ingesting L1 larvae which develop to L3 can be ingested by foxes or dogs, especially by young canids (Bolt *et al.*, 1993).

The adult female nematode in the pulmonary arteries and right ventricle of the heart sheds eggs which are then transported to the pulmonary capillaries. Eggs develop to first-stage larvae which hatch and break out into alveolar airspaces. Larvae are

coughed up, swallowed and passed in feces (Bolt *et al.*, 1992; Rosen *et al.*, 1970). The intermediate host forages on fecal material, ingesting first-stage larvae. First-stage larvae then develop to second- and third-stage (L3) larvae, which are the infective stage. The definitive hosts, wild and domestic canids, are infected by eating gastropods containing third-stage larvae (Rosen *et al.*, 1970). The L3 is digested from gastropods and/or frog tissue in the stomach and gastrointestinal tract of the definitive host. Larvae penetrate the wall of the gut and migrate to the abdominal visceral lymph nodes where they molt to fourth- and immature fifth-stage larvae (L5). The L5 is transported by the hepatic portal veins, liver and the caudal vena cava, to the right ventricle of the heart and the pulmonary arteries where they become adult parasites. The L5 is found in the pulmonary arteries and right ventricle approximately 10 days post-infection and reach maturity between 33 and 36 days after infection (Guilhon and Cens, 1973).

The prepatent period of the parasite is usually 38-57 days (Bolt *et al.*, 1994). However, Cury *et al.* (1996) showed experimentally that the prepatent period could be 28-100 days. The adult nematode lifespan is approximately equal to that of the canids. If not treated, animals can intermittently shed larvae for the rest of their lives (Bolt *et al.*, 1994; Guilhon and Cens, 1973; Rosen *et al.*, 1970).

1.7 PATHOLOGY

Angiostrongylus vasorum infection in red fox appears to be well tolerated. Infection intensities with *A. vasorum* had no effect on body condition score in red fox (Jeffery *et al.*, 2004). Concurrent infestation with sarcoptic mange may predispose the fox to develop more severe infection with *A. vasorum* (Simpson, 1996).

Angiostrongylus vasorum appears have more serious pathological effects in dogs than in the natural definitive host, the red fox (Bolt *et al.*, 1994). The immature nematodes reach the pulmonary artery and right ventricle of the heart by day 10 post infection, and cause thrombosis (Rosen *et al.*, 1970). The inflammatory process eventually extends to the tissue surrounding the pulmonary arteries which leads to foci of interstitial pneumonia. Other common pathological changes are pulmonary consolidation and fibrosis, and coagulopathy (Bourque *et al.*, 2002; Nicolle *et al.*, 2006). Larvae migrate to the airspaces and cause pulmonary haemorrhages, emphysema and focal granulomatous inflammation. Granulomas typically develop as responses to eggs and larvae (Mahaffey *et al.*, 1981; Prestwood *et al.*, 1981). In chronic infection there is pulmonary fibrosis resulting in pulmonary hypertension that can lead to right-sided congestive heart failure.

In some cases, excessive bleeding disorders due to disseminated intravascular coagulation or immune mediated thrombocytopenia can occur. Prolonged bleeding times, low platelet counts, hemoptysis, intravascular coagulation, iron deficient anemia and subcutaneous hematomas have been reported in both experimentally and naturally infected dogs (Caruso and Prestwood, 1988; Chapman *et al.*, 2004; Cury *et al.*, 2002, Gould and McInnes, 1999; Ramsey *et al.*, 1996; Schelling *et al.*, 1986; Singleton, 1994). There were also reports of dogs developing intracranial haemorrhage secondary to severe coagulation defects. These cases showed focal intracranial haemorrhages and scleral haemorrhages. Prolonged prothrombin time develops (Garosi *et al.*, 2005). A case was reported with cerebral and conjunctival haemorrhages and neurological signs which were associated with defective primary haemostasis. This was caused by extreme

deficiencies in von Willebrand factor that accompany severe bleeding disorders, which may lead to death (Whitley *et al.*, 2005).

A study involving three dogs infected with *A. vasorum*, reported clinical signs associated with hypercalcaemia (inappetence, weight loss, lethargy), and serum biochemistry indicated significant increase in ionized calcium and total calcium (Boag *et al.*, 2005). Although the authors acknowledged that hypercalcaemia is rare with *A. vasorum* infection, they suggested that hypercalcaemia has been associated with other granulomatous diseases in dogs similar to *A. vasorum* (Boag *et al.*, 2005). Another study showed 70% (41/59) of dogs infected with *A. vasorum* had high fructosamine values. Nevertheless the researchers indicated that the increased fructosamine is not a specific finding to *A. vasorum* infection, and it can occur with other diseases such as diabetes or even stress (Willesen *et al.*, 2005).

There have been several reported cases of adult *A. vasorum* causing ocular angiostrongylosis with uveitis and retinal haemorrhages (King *et al.*, 1994; Perry *et al.*, 1991; Rosenlund *et al.*, 1993).

Due to aberrant migration, adult *A. vasorum* can migrate and infect the left side of the heart and pulmonary veins. Fourteen adult *A. vasorum* were found in the left femoral artery causing swelling, haemorrhages, and finally rupture of the artery and death (Cury and Lima, 1996). First-stage larvae can migrate to the left side of the heart causing endocarditis. Subsequently they can be transported as emboli, to other organs such as the kidney, where they cause atrophy of glomeruli and focal and diffuse interstitial nephritis (Bwangamoi, 1974). Larvae can also disseminate to the liver, brain, skeletal muscles, small intestine, stomach, pancreas, spleen, thyroid gland, adrenal gland

and spinal cord. These larvae can also block capillaries causing haemorrhagic foci and granulomas (Bolt *et al.*, 1994; Perry *et al.*, 1991; Oliveira-Junior *et al.*, 2004). Another study showed 4 *A. vasorum* infected dogs had haemorrhages in the brain and spinal cord (Wessmann *et al.*, 2006).

Little is known about the infection intensities that cause severe disease or fatalities in dogs. Oliveira-Junior *et al.* (2004) reported a case of an adult dog that died suddenly due to angiostrongylosis. At necropsy they recovered a total of 316 adult nematodes: 96 nematodes from the major pulmonary artery, 114 from the branches of the pulmonary artery, 104 from the right ventricle and 1 nematode each from the pericardium sac and the urinary bladder. Various tissues were examined using the Baermann technique; first-stage larvae were recovered from skin, muscle of the diaphragm, liver, pancreas, brain, blood and cerebrospinal fluid.

Koch *et al.* (1992) reported *A. vasorum* in a dog that was severely dyspnoeic and frequently coughed blood, and had right heart enlargement which was complicated by gastric dilation. The dog was euthanized due to the poor prognosis; and during necropsy 50 mature adult *A. vasorum* nematodes were recovered within the right atrium and ventricle.

Rosen *et al.* (1970) experimentally infected 2 dogs with *A. vasorum*. One dog died at 5 ½ months post-infection, and on necropsy they recovered 185 living adult *A. vasorum* from the heart and pulmonary arteries. The other dog appeared moribund and was euthanized at 3 ½ years post-infection; at necropsy they recovered at least 200 living adult *A. vasorum*.

Martin (1989) reported another case of a 5 month old dog from Cornwall, UK (United Kingdom) that was lethargic, became dyspnoeic and died suddenly. Necropsy findings showed an enlarged rope-like mass of small nematodes which were almost completely occluding the pulmonary artery. A total of 72 adult *A. vasorum* were recovered from the mass.

1.8 CLINICAL SIGNS – DOGS

Clinical signs of *A. vasorum* infection are often not noticeable until the chronic stage months or years after infection (Bolt *et al.*, 1994). The main clinical signs have been associated with chronic pulmonary disease and cardiac failure, and less commonly neurological signs, ocular disease and sudden death (Bolt *et al.*, 1994; King *et al.*, 1994; Martin 1989; Perry *et al.*, 1991; Wessmann *et al.*, 2006). Clinical signs develop mainly due to progressive respiratory disease and cardiac failure (Bolt *et al.*, 1994). Dogs infected with *A. vasorum* show systemic signs that include depression, anorexia, gagging, coughing, weight loss, vomiting, ascites, decreased tolerance of physical activity, abdominal pain and severe dyspnea (Brennan *et al.*, 2004; Martin *et al.*, 1993). Some dogs were presented with severe dyspnea and abdominal distention, pale mucus membranes and coughing of blood (Koch *et al.*, 1992).

Neurological signs include ataxia and impaired vision (Perry *et al.*, 1991). Neurological manifestation due to cerebral hypoxia can be caused by chronic cardiac failure or the presence of embolic larvae in the brain (Perry *et al.*, 1991). In UK, 4 cases of dogs that had neurological signs including seizures, spinal cord pain and paresis were consistent with *A. vasorum* infection in the brain and spinal cord (Wessmann *et al.*,

2006). Haemorrhagic diathesis causing cerebral anemia and finally syncope and death has also been described (Patteson *et al.*, 1993).

In UK there were several case reports involving young dogs which died suddenly from *A. vasorum* infection, when adult nematodes were found in numbers sufficient to occlude the pulmonary artery (Foulkes *et al.*, 1982; Martin, 1989; Patteson *et al.*, 1987). In one such case, the young dog was passing 34,000 L1/g feces (Martin, 1989) and in another case, 280,000 L1/g feces (Martin *et al.*, 1993). In dogs, the infection can be acute, leading to death within a few days of the onset of clinical signs due to acute right-side heart failure (which is likely to be secondary to pulmonary hypertension) (Patteson *et al.*, 1993). Cury and Lima (1996) reported a dog infected with *A. vasorum* that died on day 46 post-infection, from acute hypovolemic anemia due to rupture of the femoral artery.

1.9 TREATMENT AND CONTROL

There are no anthelmintics approved for treatment of angiostrongylosis in dogs. In natural infections, levamisole, fenbendazole, ivermectin and milbemycin have been used for treatment of dogs (Conboy, 2004; Dodd, 1973; Martin *et al.*, 1993). Oral administration of levamisole at 7.5 mg/kg for two days followed by 10 mg/kg for two days was effective in the treatment of naturally infected dogs (Dodd, 1973). Soland and Bolt (1996) recorded the death of an *A. vasorum* infected dog due to hypovolemic shock after treatment with levamisole. The hypovolemic shock was presumed to be due to an anaphylactic reaction secondary to the release of nematode antigens in response to the rapid killing effects of levamisole. Levamisole is no longer recommended for use in the

treatment of canine angiostrongylosis (Soland and Bolt, 1996). Ivermectin given subcutaneously at 0.2 mg/kg and then repeated one week later, was effective in the treatment of angiostrongylosis in dogs (Martin *et al.*, 1993; Patteson *et al.*, 1993). However, it should be used with caution as the dose can be toxic to collie-type breeds (Soland and Bolt, 1996). Fenbendazole has been used orally at 20 mg/kg once or twice daily for two to three weeks (Dodd, 1973; Martin *et al.*, 1993; Patteson *et al.*, 1993) and was also reported at 50 mg/kg for 5-21 days based on the severity of the clinical signs (Chapman *et al.*, 2004). The effective dosage of milbemycin is 0.5 mg/kg weekly for a period of 4 weeks (Conboy, 2004).

With any of the treatments, post-treatment complications (dyspnea, ascites) can occur. Respiratory distress can be treated with bronchodilators and by mucolytic expectorants. Diuretics can help in edematous accumulations of body fluid and relieve the workload of the heart. Supportive therapy is recommended especially when anthelmintic treatment is to be repeated (Bolt *et al.*, 1993; Soland and Bolt, 1996).

Sanitation can limit the exposure of gastropods to feces of infected animals and may prevent the spread of infection. Infected dogs should be removed or quarantined, as the environment can remain infected for at least two years (Rosen *et al.*, 1970). Unfortunately, options directed toward control in wild canids which represent a major infection reservoir are limited.

1.10 TOOLS IN DIAGNOSIS

Clinical diagnosis of *A. vasorum* infection is based on history, signs and fecal examination. Hematological changes have been reported but were inconsistent and nonspecific (Dodd, 1973). Reported changes in evaluations of hematology and blood chemistries involved low levels of fructosamine (Willesen *et al.*, 2005), anemia and eosinophilia (Jones *et al.*, 1980) and rarely hypercalcaemia (Boag *et al.*, 2005). Changes were also seen in total differential leukocyte counts (eosinophilia, neutrophilia, basophilia, monocytosis and lymphocytosis), and reduction in haemoglobin and hematocrit. Thrombocytopenia has also been observed and immune-mediated thrombocytopenia and disseminated intravascular coagulation have been suggested as the cause (Chapman *et al.*, 2004; Cury *et al.*, 2002; Gould and McInnes, 1999; Ramsey *et al.*, 1996; Schelling *et al.*, 1986).

Radiographic findings are non specific but there have been some reports showing changes in experimentally infected dogs with diffuse increase in interstitial, bronchial and alveolar densities (Dodd, 1973). Pleural effusion fissure lines and perihilar lymphadenopathy may be visible. Enlargement of the heart and the main pulmonary artery are often visible as well (Mahaffey *et al.*, 1981). Thoracic radiographs in naturally infected dogs also show alveolar infiltration and bronchial thickening, and narrowing of the trachea (Boag *et al.*, 2004). In a dog presented with abdominal effusion, a radiograph showed pulmonary hypertension characterized by an increase in the diameter of the pulmonary arteries and right-sided cardiomegaly (Esteves *et al.*, 2004).

Most laboratory diagnoses occur by detection of first-stage larvae in feces using the Baermann technique or fecal flotation. The Baermann examination is considered the technique of choice. The specificity of the Baermann test is close to 1 unless the parasite is misdiagnosed. There are no reports regarding the sensitivity of the Baermann test, nevertheless, it is known to be reasonably high. Larvae were detected on zinc sulfate ($ZnSO_4$) fecal flotation in only 57% (8/14) Baermann-positive samples from dogs naturally infected with *A. vasorum* (Conboy, 2004). First-stage larvae are identified based on size and morphology. They are ~ 310 µm-399 µm long and possess an anterior cephalic button and a tail which terminates in a sinus wave curve with a dorsal spine. There are disadvantages with the fecal techniques; fecal flotation is often negative due to osmotic damage to the larvae. With the Baermann technique, multiple fecal samples may be required as shedding of larvae can be intermittent (Oliveira-Junior *et al.*, 2006; Patteson *et al.*, 1993).

1.11 OTHER CARDIO-PULMONARY PARASITES OF CANIDS IN NORTH-AMERICA

1.11.1 Parasites of the trachea, bronchi and lungs

Crenosoma vulpis is a metastrongylid nematode infecting the trachea, bronchi and bronchioles of dogs, foxes and bears (*Ursidae*). It occurs in the northeastern portion of North America including the Canadian provinces of Nova Scotia, New Brunswick, Prince Edward Island, Quebec, Ontario and the states of Maine and New York in the United States (Bihr and Conboy, 1999). *Crenosoma vulpis* is transmitted when the definitive host ingests snails or slugs containing infective third-stage larvae. Larvae

migrate to the lungs by way of the visceral lymphatics or via the portal system. Females deposit larvae about 19 days after infection, and the prepatent period is 18-21 days. The adult worms live 8-9 months in the final host (Jeffery *et al.*, 2004). The adult parasite females lay eggs that develop and hatch within the respiratory tract. *Crenosoma vulpis* causes chronic cough in dogs and can be mistaken for chronic allergic respiratory disease (Bihr and Conboy, 1999). Nevertheless, *C. vulpis* infection is considered to be non-fatal. At necropsy, red fox lungs that were heavily infected with *C. vulpis* showed little visible pathology, compared to lungs infected with *A. vasorum* that were severely damaged and discolored (Jeffery *et al.*, 2004). Diagnosis can be made by recovery of larvae from feces by Baermann procedure; larvae are not usually detected by fecal flotation (Bihr and Conboy, 1999). Larvae are characterized by a simple pointed tail (*A. vasorum* larvae have a kinked tail with a dorsal spine) and they are ~ 250-300 μm long (Jeffery *et al.*, 2004). A survey was conducted between the years 2000-2001 by Jeffery *et al.* (2004) in Newfoundland involving red foxes, and 87% of the red foxes were infected with *C. vulpis*. A recent survey showed that 21% of coughing dogs from Atlantic Canada were infected with *C. vulpis* (Conboy, 2004). Peak numbers of diagnoses in dogs occurred in December followed by a smaller peak in May. This correlates with the presumed peaks in terrestrial gastropod populations that occur in fall and spring due to moderate temperature and high rainfall (Bihr and Conboy, 1999).

Eucoleus aerophilus is a lungworm found in the trachea, bronchi, and bronchioles of various carnivores including dogs and cats. The life cycle may be direct or involve earthworms as intermediate hosts. The prepatent period is 21-35 days long. The eggs deposited by the female within the tracts they have made in the mucosa make

their way to the surface. The freed eggs are then coughed up, swallowed, and passed in feces (Bowman, 2003). Infection in dogs and cats is usually asymptomatic but sometimes nasal discharge and a mild cough can be observed. In farmed foxes the infection may be more severe with heavy coughing, wheezy respiration, weakness, poor growth and death due to bronchopneumonia. Diagnosis is based on identifying eggs in feces or tracheal mucus. Fenbendazole was a successful treatment for *E. aerophilus* infection (Bowman, 2003).

Oslerus (*Filaroides*) *osleri* are very small nematodes that are found in nodules that tend to be located close to the bifurcation of the trachea. The males are only 4-7 mm long and the females are only slightly longer. The parasite stimulates the formation of fibrous nodules in the trachea and bronchi of canids. A dog will exhibit chronic cough and exercise intolerance (Bowman, 2003). The stage passed in the feces is a first-stage larva which is virtually indistinguishable from the first-stage larva of *Filaroides hirthi*. Larvae are best detected in fecal samples by using direct smears or zinc-sulfate centrifugal flotation. Larvae passed in feces are characterized by having a tail that has a constriction just anterior to its tip, which gives the very tip of the tail a kinked appearance. This parasite is considered to be distributed globally but is infrequently diagnosed in dogs (Outerbridge and Taylor, 1998). The life cycle of *O. osleri* is direct. Dogs are probably commonly infected as puppies via transmission of larvae in sputum by licking and cleaning of the mother or through regurgitated food. The most obvious sign of infection with *O. osleri* is a dry cough that is precipitated by exercise. There are usually no signs of serious disease until the nodules are sufficiently enlarged to cause obstruction of air flow. Some dogs present with a persistent cough over a year long

period (Bowman, 2003; Urquhart *et al.*, 1996). The diagnosis of infection is confirmed by viewing the nodules in the trachea with a bronchoscope, and the presence of the fibrous nodular projections into the lumen of the trachea and bronchi (Bowman, 2003).

1.11.2 Parasites of the respiratory parenchyma

Filaroides species are directly infective as first-stage larvae and require no development outside the body of the host or in an intermediate host. *Filaroides hirthi* is an infrequent cause of severe, potentially fatal, respiratory tract disease of immunosuppressed dogs (Bowman, 2003). These are very small metastrongyloid nematodes, deeply buried in the parenchyma of the lungs. Larvae occurred in the mesenteric lymph nodes, intestinal walls, and liver of two experimentally immunosuppressed beagles (Genta and Schad, 1984). Hirth and Hottendorf (1973) were the first to describe *F. hirthi* in a laboratory research beagle in the USA. There have been reports of subclinical infections in laboratory research beagles (Hirth and Hottendorf, 1973) and fatal respiratory tract disease also has been associated with severely stressed animals (Genta and Schad, 1984).

Paragonimus kellicoti is a lung fluke, class Trematoda, family Troglotrematidae. This parasite occurs in pairs in cysts in the lungs of cats and dogs and various wild carnivores in the eastern United States, mainly in the drainage systems of the Mississippi River and Great Lakes. The large eggs of the fluke are swept up the tracheobronchial tree, swallowed and passed in feces (Bowman, 2003). If the eggs are in water, miracidia develop and hatch in two weeks and enter a snail, in which cercariae develop through one sporocyst and two redial stages. Cercariae leave the snail and encyst as

metacercariae in crayfish. Dogs and cats become infected by ingesting crayfish containing the encysted cercariae or by eating animals that have recently fed on crayfish. The young flukes migrate to the peritoneal cavity for a week to 10 days and then enter the pleural cavity through the diaphragm. The flukes begin to enter the lungs around two weeks after infection. By three weeks after infection, there will typically be pairs of flukes found together in the lung tissue (Bowmann, 2003). The adult flukes are about 6.35 mm long and live within cysts within the lungs. Clinical signs tend to be mild involving cough. Occasionally, sudden death may occur due to pneumothorax from the rupture of pleural cysts (Bowman, 2003).

1.11.3 Parasites of nasal mucosa and sinuses

Eucoleus boehmi is a nematode which belongs to the superfamily Trichinelloidea. *E. boehmi* has been reported in North America, South America and Europe. The adult nematodes are ~ 15-40 mm long and found in the mucosa of the nasal sinuses. The adults are ~ 0.1 mm in diameter and this causes them to appear as very fine threads when they are removed from the mucosa at necropsy (King *et al.*, 1990). The life cycle of *Eucoleus boehmi* is not known; some related species of *Eucoleus*, such as *Eucoleus aerophilus* which is in the lungs of dogs and cats, require an earthworm intermediate host. Other species of *Eucoleus* have direct life cycles (King *et al.*, 1990). There have been several cases where this parasite has caused disease in canids. King *et al.* (1990) initially examined a dog that was found positive for this parasite because of a complaint of chronic nasal discharge.

1.11.4 Parasites of the heart and pulmonary vessels

Angiocaulus gubernaculatus belongs to the family Angiostrongylidae. The parasite infects the pulmonary arteries and right ventricle of wild canids and mustelids (Ubelaker, 1986). *Angiocaulus gubernaculatus* was recovered from an island fox (*Urocyon littoralis*) during necropsy to determine the cause of death (Faulkner, 2001), and in the California badger (*Taxidea taxus*) (Ubelaker, 1986). The life cycle of the parasite has not been studied; it is presumed to resemble that of other parasites in the same family and therefore would likely involve gastropod intermediate hosts (Rosen *et al.*, 1970). Nothing is mentioned in the literature about clinical signs or pathology. Costa *et al.* (2003) considered the genus *Angiocaulus* to be a synonym of *Angiostrongylus*. *Angiocaulus gubernaculatus* closely resembles *A. vasorum* morphologically. The species are differentiated based on slight differences in spicule size and bursal ray morphology in the male nematode (Ubelaker, 1986).

Dirofilaria immitis, the North American heartworm, belongs to the superfamily Filarioidea and it infects the pulmonary artery and right ventricle of the heart of canids, cats, ferrets (*Mustela putorius furo*) and sea lions (*Zalophus californianus*). Adult *D. immitis* are easily differentiated from *A. vasorum* based on size (*D. immitis* are up to 30 cm long). Mosquitoes serve as the intermediate host and ingest microfilariae when feeding on blood from an infected dog. The microfilaria develops into an infective L3 in about 14 days. When the mosquito again feeds on a dog, the infective third-stage larva is injected into the skin (Bowman, 2003). The third-stage larva moults to the fourth-stage within 48 h. The fourth-stage larva migrates through the dog's tissues, eventually reaching the pulmonary artery where it moults and matures to the adult stage. The

female nematode deposits microfilariae into the blood stream. The prepatent period is 6-9 months (Bowman, 2003).

Dogs with low parasite burdens are usually asymptomatic, however, high worm burdens can cause vascular damage resulting in a decrease of lumen size which causes hypertension that can lead to right-side congestive heart failure (i.e. cor pulmonale). Cor pulmonale usually takes 3-5 years of infection to develop. Dogs fatigue easily, cough, and appear unthrifty (Bowman, 2003; Murdoch, 1984). Blood backs up in the liver and other parts of the body, causing general congestion and degeneration. Liver failure is another syndrome associated with *D. immitis* infection, when dogs are exposed to tremendous numbers of third-stage larvae over short periods. Hemolysis will develop due to the presence of 100 or more developing nematodes in the pulmonary artery and right ventricle of the heart. The presence of high numbers of *D. immitis* will interfere with the heart valve function and lead to massive hemolysis of red blood cells. A dog will die within 48 h without emergency surgery to remove nematodes from the tricuspid area (Bowman, 2003).

Treatment of adult *D. immitis* includes melarsomine with a two injection protocol that is used in dogs with low risk of post-treatment thromboembolism: 1 dose which is repeated in 24 h. A three injection protocol is used in dogs with high risk of post-treatment thromboembolism: 1 dose followed in 4-6 weeks by second dose, and a third dose is given 24 h after the second treatment. Microfilaremia can be eliminated by treatment with milbemycin or ivermectin 4-6 weeks after adulticide treatments. Canine heartworm infection is preventable, despite the inherent susceptibility of dogs (Nelson *et al.*, 2005). The most commonly used heartworm preventions are the macrocyclic

lactones which include monthly oral administration of ivermectin or milbemycin oxime, and monthly topical application of moxidectin or selamectin (Nelson *et al.*, 2005).

Diagnosis by detection of microfilariae is performed using the Modified Knotts and Filter test. The method of choice to diagnose *D. immitis* is the antigen ELISA test for detecting circulating heartworm antigen and it is performed in dogs 7 months of age and older. This antigen test is very accurate with 97-98% sensitivity (Datz, 2003; Nelson *et al.*, 2005). Nevertheless, false-negative results occur most commonly when infections are light, female nematodes are still immature, only male nematodes are present and/or the test kit has not been warmed to room-temperature. False-positive results may occur due to technical error, such as inadequate washing steps or delay in reading the test (Nelson *et al.*, 2005). However, the heartworm antigen test is the most reliable method of diagnosing infection with adult *D. immitis*. Moreover, the antigen test is the most reliable method of confirming the success of adulticide therapy. If all adult female nematodes have been destroyed, heartworm antigen should become undetectable by 6 months post-treatment. The follow-up antigen test can also help differentiate between a persistent infection and re-infection if antigenemia is detected again at a later date (Nelson *et al.*, 2005).

1.12 HYPOTHESES AND OBJECTIVES OF THIS STUDY

1. **Hypothesis –** Dogs infected with *A. vasorum* will have a humoral immune response to the parasite. The antibodies produced can potentially be detected by an ELISA test and this can be used as a means to diagnose *A. vasorum* infected dogs.

   Objectives – Develop an ELISA test (Indirect ELISA) using *A. vasorum* adult somatic antigen that will detect antibodies produced by dogs in response to infection with French Heartworm. Determine if the test cross-reacts with other common canine helminth parasites in the Atlantic region.

2. **Hypothesis** – Adult nematodes dwelling in the pulmonary artery and right ventricle of an infected dog will release antigens. These antigens can be detected by immunological means and used as the basis for a diagnostic test which will be superior to fecal examination with respect to detection sensitivity.

   Objectives – Using rabbit polyclonal antisera prepared against crude *A. vasorum* adult somatic antigen, a sandwich ELISA will be developed that will detect the parasite antigen(s) released into circulation in infected dogs.

3. **Hypothesis** – Identifying and obtaining large pure quantities of the circulating *A. vasorum* antigen(s) will allow the production of specific polyclonal and monoclonal antibodies that will improve test sensitivity when used in the sandwich ELISA test.

   Objectives – Construct a cDNA library from adult *A. vasorum.* The cDNAs have no intron sequence and can thus be used to express the encoded protein in *E. coli.*

Since the cDNA is derived from mRNA, cDNA represents the transcribed parts of the genome. This will be the first study to produce a cDNA library from *A. vasorum*, and to sequence genes coding for immunoreactive proteins. Potentially these proteins can be introduced into a mouse or rabbit for production of specific monoclonal or polyclonal antibodies, respectively, to be used in the sandwich ELISA.

4. **Hypothesis –** It is necessary to produce a recombinant protein from the *A. vasorum* cDNA library for production of specific monoclonal or polyclonal antibodies to be used in the sandwich ELISA and to generate sufficient amounts of reagents and increase sensitivity

   Objectives – Glutathione-S-transferase (GST) fusion protein system is widely used. The insert of interest encoding for a vitellogenin protein homologue (from the *A. vasorum* cDNA library immunoreactive clones) cloned into the pGEX will be produced as a fusion to GST. This will allow subsequent proteolysis necessary to obtain pure protein of interest. The pure protein will be introduced into a mouse or a rabbit for production of monoclonal or polyclonal antibodies respectively. These antibodies can be produced in unlimited amounts and be used in the sandwich ELISA, potentially increasing sensitivity of the test.

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CHAPTER 2: SEROLOGIC DIAGNOSIS OF *ANGIOSTRONGYLUS VASORUM* USING ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

2.1 ABSTRACT

Angiostrongylus vasorum, French Heartworm, is a metastrongylid nematode infecting the pulmonary arteries and right heart of domestic and wild canids. It is the cause of fatal cardiopulmonary disease, bleeding disorders and damage to other organs (kidneys, liver, eyes and brain). Definitive hosts are infected by the ingestion of an infected slug or snail intermediate host. Dogs may not develop signs of clinical disease until months or years after infection. Fecal diagnosis of *Angiostrongylus vasorum* may lack sensitivity due to intermittent shedding of larvae. In this study, serological tests were developed to detect infection with *A. vasorum*, similar to those used for the diagnosis of the North American Heartworm (*Dirofilaria immitis*). An initial attempt to use an enzyme linked immunosorbent-assay (ELISA) to detect antibodies to the parasite was confounded by significant cross-reactivity to antigens of *Crenosoma vulpis*, a common metastrongylid lungworm parasite of canids in Atlantic Canada. Therefore a sandwich ELISA was developed to detect circulating antigens of the parasite. A positive cut-off value and test sensitivity and specificity were determined by two-graph receiver operating characteristic curve (ROC) analysis using sera from 24 Baermann positive *A. vasorum* dogs from Newfoundland and sera from 52 Baermann negative *A. vasorum* dogs from non endemic areas. An optical density of 0.19 was used as the positive cut-off and the test specificity was 100% with a sensitivity of 92%. Furthermore, the test detected *A. vasorum* antigens in sera from 10/10 experimentally infected dogs by 4 weeks post-infection. In addition, the test did not cross-react with 30

sera samples from dogs infected with the lungworm, *Crenosoma vulpis*. A fecal survey of Newfoundland dogs suffering from chronic cough indicated that 10% (24/239) were infected with *A. vasorum*. The ELISA detected circulating antigen in 19% (45/239) of these dogs indicating that fecal diagnostics may have missed about half of the infected dogs.

2.2 INTRODUCTION

Angiostrongylus vasorum (French Heartworm) is a metastrongylid nematode parasite, with an adult stage which resides in the pulmonary artery and the right ventricle of the heart in wild and domestic canids. Animals are infected by ingesting gastropod intermediate hosts (slugs and snails) (Bolt *et al.*, 1994). *Angiostrongylus vasorum* infection can cause fatal cardiopulmonary disease in dogs (Bolt *et al.*, 1994). Clinical signs develop due to a progressive respiratory disease which can potentially result in cardiac failure. Nevertheless, dogs may be infected for prolonged periods before showing any clinical signs (Bolt *et al.*, 1994). The prepatent period of the parasite is usually 38-57 days (Bolt *et al.*, 1994). However, Cury *et al.* (1996) showed experimentally that the prepatent period could be from 28-100 days. The life span of an adult *A. vasorum* can be as long as its infected host (Conboy, 2004).

Diagnosis of *A. vasorum* is a key factor that will allow early treatment of infected dogs before serious cardiopulmonary disease develops. Currently, *A. vasorum* infection is diagnosed by detection of first-stage larvae in feces using the Baermann technique or fecal flotation; diagnostic investigation is only initiated after dogs show clinical signs of disease. The Baermann test is considered the technique of choice as larvae were

detected on fecal flotation in only 57% (8/14) of Baermann positive samples from dogs naturally infected with *A. vasorum* (Conboy, 2004). However, the Baermann fecal technique itself can be inconsistent. Multiple fecal samples may be needed due to periodic shedding of larvae (Oliveira-Junior *et al.*, 2006; Patteson *et al.*, 1993).

Serological techniques have been used in the diagnosis of various nematode infections (Agneessens *et al.*, 2001; Eysker and Ploeger, 2000; Grieve *et al.*, 1981). Agneessens *et al.* (2001) developed an enzyme linked immunosorbent assay (ELISA) test for detecting *Ostertagia ostertagi* in cattle. The ELISA was based on polyclonal rabbit antibodies which recognized *O. ostertagi* excretory/secretory antigens (Agneessens *et al.*, 2001). Eysker and Ploeger (2000) reviewed the various diagnostic techniques employed for the detection of trichostrongyle infection in ruminants including the use of serology. The antigen ELISA test using crude parasite antigens has been used for diagnosis of *Haemonchus contortus*, *Cooperia oncophora* and *O. ostertagi* and appears to show promise as a parameter for herd health monitoring. An ELISA test to detect circulating nematode antigen has been developed for the diagnosis of the North American Heartworm, *Dirofilaria immitis* (Grieve *et al.*, 1981; Weil, 1989; Weil *et al.*, 1985). The test was found to be highly specific and sensitive, and is the best choice in diagnosis of this disease (Courtney and Zeng, 2001; Datz, 2003). The current study was an attempt to develop a serological test, similar to those used for the diagnosis of the *D. immitis* to detect infection with the French Heartworm.

An ELISA test is capable of quickly determining whether antibodies have been developed against the parasite or for detecting antigens released from the parasite during infection. The indirect ELISA is used to detect circulating antibodies against *A.*

vasorum in infected dogs. Infection with *A. vasorum* induces the host to produce antibodies to nematode antigen(s) which can be detected by ELISA. Detection of a host antibody response may be superior to the existing fecal examination with respect to detection sensitivity for this parasite. The antigen of interest is directly bound, or coated onto a microtiter plate. Antibodies present in the sera of the infected dogs react with the antigen and are detected using an enzyme labeled anti-globulin and enzyme substrate. The bound antigen can be detected and quantified by incubating the plate with antibodies specific to the antigen of interest. The enzyme activity is a measure for the amount of bound antibody-enzyme conjugate and therefore for the amount of antibody present. The sandwich ELISA is used to detect circulating antigens released by *A. vasorum* during infection in dogs. The microtiter plate is first coated with an antibody specific to the antigen(s) of interest and this same antigen present in the dog serum is detected with the use of another enzyme labeled antibody specific to the antigen of interest. The objective for this study was to develop an ELISA to detect *A. vasorum* infection in dogs.

2.3 MATERIALS AND METHODS

2.3.1 Antigen preparation

The antigen used for rabbit immunization was prepared from *A. vasorum* adult nematodes that were collected from trapped foxes from Newfoundland. Approximately 500 pieces of worms were counted and washed with saline. To prepare the antigen the nematodes were mixed with saline and placed into a tube, allowed to settle and were ground in a 7 ml Tenbrock grinder. One half milliliter of ground nematodes was mixed

with one half milliliter of glass beads (425-600 mm diameter) placed into a minibead beater™ (Biospec products) apparatus and shaken twice at 5000 rpm for 30 sec. The tubes were briefly spun in a microcentrifuge for 2 sec at 11,750 × g (Eppendorf centrifuge model 5415C with standard rotor) and left to settle for 4 min. Supernatants were collected and pooled. The beads were washed by vortexing for 2 sec with 0.5 ml saline, centrifuged for 2 sec at 16,000 × g and the supernatant collected. Nematode preparations were pooled and assayed for total protein concentration using a Micro-BCA protein assay kit (Pierce) according to the manufacturer's instructions.

2.3.2 Immunization of mice and monoclonal antibody preparation

The following is a modification of the technique reported by Kohler and Milstein (1975) and Newbound *et al.* (1993). Female BALB/c mice were immunized with crude antigen that was prepared as previously described. The antigen was mixed 1:1 with double concentrated adjuvant (MPL/TDM, Sigma) to give an inoculum of 250 µg of protein/ml. Mice were immunized intra-peritoneally with 200 µl which contained 50 µg of protein. Three immunizations were given over the period of 3 months. Four days after the third immunization, the mice were euthanized. The spleen was removed, passed through a tissue sieve to produce a single cell suspension which was then fused with myeloma cells. Supernatant fluid from hybridomas was tested by indirect ELISA and positive clones were subcultured by limiting dilution, retested and expanded. Tissue culture supernatant was collected and stored at -20°C.

2.3.3 Immunization of rabbits and polyclonal antibody preparation

Female New Zealand White rabbits were immunized with the crude antigen derived from foxes and prepared as previously described (section 2.3.2). The antigen was mixed 1:1 with adjuvant (MPL/TDM/CWS, Sigma) to give an inoculum of 250 µg of protein in 0.6 ml. This was administered subcutaneously and evenly distributed to the hind legs and the neck. Three immunizations were given over a 3 month period. The specific antibody titer was determined by ELISA using a crude antigen concentration of 2 µg/ml in coating buffer and 100 µl per well to coat the microtitre plate. Two weeks after the third immunization, the rabbits were euthanized and a final bleed was performed. Sera were collected and stored at -20°C.

2.3.4 Affinity chromatography purification

Immunoglobulin was purified from rabbit serum using affinity chromatography with protein G-Sepharose®Fast Flow (Pharmacia) according to manufacturer's specifications. Protein concentration of the polyclonal antibody and monoclonal antibody was determined using the Micro-BCA protein assay kit (Pierce).

2.3.5 Biotinylation of purified polyclonal and monoclonal antibodies

A biotinylation protocol from Sigma was used. A stock of N-hydroxysuccinimide biotin (Sigma) at 10 mg/ml in DMSO (dimethyl sulphoxide) was prepared and was mixed with 2 mg/ml antibody in sodium borate (0.1 M, pH 8.8, Sigma) to give a 100 µg biotin/mg of antibody mixture. The mixture was incubated for 4 h at room-temperature with stirring. Ammonium chloride (1 M, NH_4Cl, Sigma) was added to the antibody

solution at a concentration of 20 µl/250 µg of biotin (to stop the biotinylation reaction), incubated for 10 min at room-temperature followed by dialysis (6000-8000 MW cut off, Fisher) against saline for 48 h at 4°C.

2.3.6 Indirect ELISA

The crude antigen preparation was diluted to 2 µg/ml in a carbonate: bicarbonate coating buffer ($Na_2CO_3/NaHCO_3$, pH 9.6, Fisher) and was dispensed into a polystyrene microtitre plate (Dynex,VWR) at 100 µl per well. The *A. vasorum* antigen was allowed to bind to the wells overnight at 4°C. The plate was washed 3 x with PBST (Phosphate buffered saline, 0.05% Tween-20) at pH 7.4 and then blocked with 3% bovine serum albumin in PBST at 200 µl per well and incubated for 1 h at room-temperature. The plate was washed 3 x. Following the washes, sera from *C. vulpis* and *A. vasorum* infected dogs were added in 3 dilutions of 1:100, 1:250 and 1:500 with PBS-1% BSA. All dilutions were added to the plate at 100 µl per well and incubated for 1 h at 37°C. Following incubation the plate was washed 3 x. Horseradish peroxidase anti-dog IgG conjugate diluted at 1:1000 in PBS-1% BSA was added at 100 µl per well and incubated for 1 h at 37°C. The plate was washed 3 x and a substrate containing 0.2% hydrogen peroxidase (30% stock) and ABST (2,2'-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid], Roche) was added at 100 µl per well and the plate was incubated at 37°C for 1 h. The plate was read at 405 nm with reference wavelength of 490 nm and the optical density (OD) values were obtained.

2.3.7 Sandwich ELISA using monoclonal antibody

The same procedure used to establish the polyclonal antibody sandwich ELISA was applied to the monoclonal antibodies to detect crude antigen in sera from dogs. The monoclonal antibodies were used in 3 different combinations. First, polyclonal rabbit antiserum was used as the capture antibody and a monoclonal antibody as the detecting antibody. Secondly, an IgG affinity purified monoclonal antibody was used as a capture antibody and the rabbit polyclonal was used as the detecting antibody. The third combination used an affinity purified monoclonal as the capture antibody and biotinylated affinity purified monoclonal as the detecting antibody. The conjugates used were chosen according to the monoclonal isotype and were either Goat anti-Mouse IgG or IgM. A peroxidase labeled streptavidin conjugate was used as before for all experiments utilizing biotinylated detection antibodies.

2.3.8 Sandwich ELISA using polyclonal antibody

A polystyrene plate (Dynex, VWR) was coated with 100 µl affinity purified rabbit polyclonal antibody (capture antibody) at 5 µg/ ml in coating buffer. The plate was incubated at room-temperature for 1 h and then overnight at 4°C. The plate was washed 3 x with PBST at pH 7.4, blocked with a 3% bovine serum albumin (BSA) blocking solution in PBST at 200 µl per well and incubated for 1 h at room-temperature. Following the blocking step the plate was washed 3 x with PBST, and the crude antigen in 100 µl in antibody buffer (1% BSA in PBST) buffer was titrated using doubling dilutions starting at 5 µg/ml. The plate was incubated for 1 h at 37°C and washed 3 x with PBST. The biotinylated detecting antibody was diluted 1/50 in

antibody buffer (1% BSA in PBST), dispensed at 100 µl per well and the plate was incubated at 37°C for 1 h. The plate was washed 3 x with PBST and 100 µl of peroxidase labeled streptavidin conjugate (CEDARLANE laboratories) diluted to 1/2000 (1% BSA in PBST) was dispensed into each well. The plate was incubated for 1 h at 37°C. The plate was then washed with PBST and 100 µl per well of a 0.2% hydrogen peroxide (30% stock) substrate containing ABST (2,2'-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid], Roche) was added and the plate was incubated at 37°C for 1 h. The plate was read at 405 nm with reference wavelength of 490 nm and the optical density (OD) values were obtained.

After establishing the validity of the sandwich ELISA using the crude antigen, dog sera were evaluated in an attempt to detect circulating antigens. A microtitre plate was coated with the affinity purified rabbit polyclonal antibody (5 µg/ml) at 100 µl per well. The plate was incubated for 1 h at room-temperature and then overnight at 4°C. After washing, the plate was blocked with 3% BSA in PBST at 200 µl per well and incubated for 1 h at room-temperature. Following incubation, the plate was washed and individual dog serum diluted 1/4 with antibody buffer (1% BSA in PBST) was dispensed into the microtitre wells. The plate was incubated at 37°C for 1 h and then washed. The biotinylated antibody was applied at 1/50 dilution (1% BSA in PBST) and the plate was incubated at 37°C for 1 h. Following incubation, the plate was washed and a peroxidase labeled streptavidin conjugate diluted 1/2000 (1% BSA in PBST) was dispensed at 100 µl per well. The plate was incubated for 1 h at 37°C, washed and a substrate containing H_2O_2 and ABST was dispensed at 100 µl per well and the plate was incubated at 37°C for 1 h.

2.3.9 Source of Dog Sera

Sera were collected from 52 dogs from areas non endemic for *A. vasorum*; 29 dogs from Minnesota, and 23 dogs from Atlantic Veterinary College (AVC), Prince Edward Island, which were admitted for orthopedic and elective surgeries. Twenty-four sera samples from dogs with natural *A. vasorum* infection from Newfoundland (Baermann positive) that had clinical signs of respiratory disease were also tested. Additional positive sera was obtained from 10 laboratory research beagles (10-12 months of age, 5 intact females, 5 neutered males; Marshall Farms, North Rose, New York, USA) that were experimentally infected with *A. vasorum* and served as untreated controls in a separate drug efficacy study. Sera were collected at weeks 2, 4, 6, 8, 10 and 11 post-infection. Fecal larval counts were monitored at 10, 11, 12, 13 and 14 weeks post-infection. Necropsies were performed on the dogs at 14 weeks post-infection and nematode counts were done. Furthermore, sera from 30 dogs were collected from areas non endemic for *A. vasorum* that were positive on Baermann fecal examination for *Crenosoma vulpis* to test for possible cross-reaction on sandwich ELISA. *Crenosoma vulpis* is a common lung worm of canids in Atlantic Canada which also causes coughing in dogs. Finally, 239 sera and fecal samples were collected from two surveys in Newfoundland in 1998-2000 and 2000-2002 from dogs with clinical signs of cardiopulmonary disease (Table 2.1).

2.3.10 Experimental infection of dogs

First-stage larvae of *A. vasorum* were obtained from Diagnostic Services at AVC from a naturally infected dog from Newfoundland. The larvae were recovered by Baermann technique and fed to aquatic snails (*Biomphalaria glabrata*) on lettuce at a level of 400-800 L_1/snail. Snails were digested 3-4 weeks later in an acid-pepsin solution (10.0 g pepsin, 13.3 ml concentrated hydrochloric acid, 1670 ml distilled water) for 2 h at 37°C. Infective third-stage larvae (120 L_3) were recovered and divided into 3 gelatin capsules which were given orally, to a 12 month old intact female laboratory research beagle. The dog began shedding larvae at 51 days post-infection. The larvae shed by this dog over a period of 2 months were used to infect *B. glabrata* for use in a drug efficacy study. The 10 untreated control group beagles (5 males, 5 females) were given 150 L_3 in gelatin capsules (administered as above).

2.3.11 Statistical analysis

A two-graph receiver operating characteristic curve (ROC) was used to determine the optimal cutoff value for the sandwich ELISA. The ROC analysis is a representation of the trade-offs between false negative and false positive rates (Altman, 1991; Dohoo *et al.*, 2003). When testing the dog sera we needed to establish a cut-off (absorbance value) that would indicate positive or negative status. The cut-off point was established by ROC analysis, using the optical density parameters from 52 *A. vasorum* negative dogs and 24 *A. vasorum* Baermann positive dogs. Confidence intervals for sensitivity and specificity were computed by the "plus four" method (Moore and McCabe, 2006).

Kappa and McNemar's test statistics (Dohoo *et al.*, 2003) were used to quantify the agreement between the Baermann and sandwich ELISA tests in the two surveys that were conducted in years 1998-2000 and 2000-2002.

2.4 RESULTS

2.4.1 The use of indirect ELISA to detect antibodies to *A. vasorum* in sera of dogs infected with *A. vasorum* and *C. vulpis*

Sera from dogs infected with both *A. vasorum* and *C. vulpis* reacted in the indirect ELISA using *A. vasorum* antigen. Values for sera from *C. vulpis* infected animals were very similar to those of the *A. vasorum* infected dogs, indicating significant cross-reaction on the indirect ELISA test (Table 2.2).

2.4.2 Sandwich ELISA using monoclonal antibody

Three experimental combinations were explored. The first using the rabbit polyclonal antibody as capture antibody, and the monoclonal antibody as the detecting antibody, yielded very low optical densities when using dog sera (Figure 2.1, Table 2.3). The second combination using the IgG affinity purified monoclonal as the capture antibody and polyclonal as the detecting antibody produced slightly higher optical densities but were still considered to be low for *A. vasorum* positive sera (Figure 2.1, Table 2.3). The third combination using the monoclonal as the capture antibody and the biotinylated monoclonal as the detection antibody again produced low optical densities even when using positive *A. vasorum* dog sera (Figure 2.1, Table 2.3). The 3 different combinations had limited success in detecting the *A. vasorum* antigen. The monoclonal

antibodies produced very low optical densities (OD) with the crude antigen and even lower ODs with the dog sera.

2.4.3 Sandwich ELISA using polyclonal antibody

Rabbit polyclonal antiserum was able to function both as capture antibody and as detecting antibody. Titration of the crude antigen (doubling dilutions starting at 5 µg/ml) in the sandwich ELISA demonstrated detection even when the crude antigen was in very low dilutions (Figure 2.1). Preparation of this crude antigen was used for determination of specificity and sensitivity and the titration of the crude antigen curve was included as a positive control. Compared to the monoclonal combinations, the polycloal-polyclonal antibody combination could detect *A. vasorum* positive sera with high optical densities (Table 2.3).

2.4.4 Sensitivity-Specificity and determination of cut-off values

Baermann fecal negative sera from Minnesota and PEI dogs were used as negative samples to establish the sensitivity-specificity and a test cut-off value. The dogs (15 males and 20 females, sex unknown for 17 dogs) ranged in age from 2 to14 years. The sandwich ELISA optical density readings from these dogs ranged from 0.011 to 0.162 (Mean= 0.067, SD= 0.041) (Table 2.4). Baermann *A. vasorum* positive samples were represented by 24 dogs from Newfoundland and the optical densities ranged from 0.03 to 0.463 (Mean= 0.246, SD= 0.089) (Table 2.4). The ROC analysis showed the optimal OD cut-off value (assuming equal costs of false positive and false negative errors) to be in the range of 0.162-0.193; the single value 0.19 was chosen (Figure 2.2).

At this cut-off value, all 52 samples from non endemic area dogs were negative and 22 naturally infected dogs from Newfoundland were positive using the sandwich ELISA. This gives a sensitivity of 0.92 (95% CI: 0.73, 0.99) and a specificity of 1 (95% CI: 0.94, 1) (Table 2.4). Furthermore, all 30 sera samples from *C. vulpis* infected dogs (14 males and 11 females: 1-13 years, Mean age=3.76; information was unavailable for 5 dogs), tested negative using the sandwich ELISA test (Min OD=0.01, Max OD=0.09, Mean=0.05, SD=0.025).

2.4.5 Baermann fecal survey sera samples

After establishing the cut-off, and determining that there was no cross-reactivity with sera from animals infected with *Crenosoma vulpis*, the sandwich ELISA was used to evaluate sera collected in two fecal surveys (1998-2000 and 2000-2002) of *A. vasorum* infection in dogs from Newfoundland that had clinical signs of cardiopulmonary disease (Table 2.5). ELISA used cut-off of 0.19, but no changes in the results would occur with cut-offs within the optimal range 0.161-0.193 determined earlier. Dogs ranged in age from 0.5 to 18.5 years (Mean=6.54) with 133 females (83 intact, 50 spayed) and 106 males (84 intact, 22 neutered). Thirty-one breeds were represented, the most common being the beagle (54 dogs) and the Labrador retriever (39 dogs). Twenty-four of the samples (24/239=10%) were positive for *A. vasorum* on Baermann fecal examination. The sandwich ELISA test detected circulating antigen(s) of *A. vasorum* in sera from 45 dogs (45/239=19%). The dogs ranged in age from 1 to14 years (Mean=5.113, SD=2.957) and were comprised of approximately equal numbers of males and females (22 females and 23 males). Twenty-two of the 182 dogs

(22/182=12%) from the 1998-2000 fecal survey were positive for *A. vasorum* on the sandwich ELISA and 5 (5/182=2.7%) were positive on the Baermann test; the difference in prevalence of infection between the tests was strongly significant (P<0.001). Comparison of the Baermann exam and the ELISA for this survey yielded a kappa statistic of 0.34 indicating low agreement between the two tests. Twenty-three of the 57 dogs (23/57=40%) from the 2000-2002 fecal survey were positive for *A. vasorum* on the sandwich ELISA test and 19 dogs (19/57=33.3%) were positive on the Baermann test. Comparison of the two tests in this survey gave a kappa statistic of 0.70, demonstrating closer agreement between the two tests than in the 1998-2000 survey. The higher prevalence of the ELISA was not statistically significant (p=0.29). The seasonal distribution was similar for both methods of diagnosis; 83% of the Baermann positive samples occurred from October through February while 84% of the sandwich ELISA positive samples occurred from October through March. There were no positive results by either method from samples collected during July, August or September (Figure 2.3).

2.4.6 Experimentally infected dogs

The 10 beagles began shedding *A. vasorum* larvae by 9 weeks PI (post infection). Larval counts at 10 weeks PI ranged from 5 larvae/g feces (LPG) to 656 LPG (Mean=225; SD=208). At week 14 PI the fecal larval counts ranged from 446 to 21098 LPG (Mean=4326; SD=5870). There was a general trend of increased larval shedding in the group of dogs throughout the sampling period. However, larval shedding patterns for individual dogs were noisy with some dogs showing as much as a 10-fold increase or decrease in LPG levels between succeeding sampling weeks. The nematode counts

ranged from 41 to 105 adult *A. vasorum* (Mean=74.2; SD=18.8) recovered from the right ventricle and pulmonary arteries of the dogs at necropsy. Pre-infection sera sandwich ELISA OD values were low (range 0.03-0.12). ELISA results indicated that 50% of the dogs (5/10; OD range 0.20-0.27) were positive by the second week post-infection. All 10 dogs were ELISA positive at 4 weeks PI (OD range 0.20-0.27) and remained positive throughout the study with individual OD values of 0.20-0.38 (Table 2.6). Mean OD values for the 10 dogs were greater than 0.30 from week 6.

2.5 DISCUSSION

In this study, diagnosis of *A. vasorum* by the the detection of antibodies to *A. vasorum* using the indirect ELISA was confounded by significant cross-reactions to *C. vulpis*, making the use of such a test unlikely to be successful and indicating that the antigen ELISA test would be more useful. At first monoclonal antibodies were used in the sandwich ELISA. The antibodies were developed from immunized mice, using spleen cells fused with myeloma cells. The hybridomas were tested by ELISA and positive clones were subcloned and retested. Three monoclonal IgM isotypes were developed and one IgG. They were all tested on the sandwich ELISA test in 3 different combinations. The monoclonal antibodies produced low optical densities with the crude antigen and even lower optical densities with dog sera. One possible explanation is that the antigen contained too much non-relevant protein (crude antigen and dog sera were used) as compared to the target protein. Another problem could be with affinity of the monoclonal antibodies. Low or weak affinity will produce weaker results if the monoclonal antibody does not bind strongly to the epitope. Due to the low signals that

were produced from the monoclonal antibodies, the study continued focusing on the rabbit polyclonal antibody and its use in the sandwich ELISA test.

The sandwich ELISA test using the rabbit polyclonal antisera had specificity of 100% and sensitivity of 92% at an optical density cut-off value of 0.19 (the cut-off was selected to obtain the maximum specificity and eliminate false positives but retain high sensitivity). The test detected antigen in sera from 10/10 experimentally infected dogs by 4 weeks post-infection (5 weeks earlier than fecal larval shedding was detected using the Baermann technique). In addition, the test did not cross-react with any of the 30 sera samples from dogs infected with the lungworm, *Crenosoma vulpis*.

In the 1998-2000 Newfoundland survey of dogs with signs of cardiopulmonary disease, there was a fairly poor agreement between Baermann and ELISA tests, due to a much lower number of positives on the Baermann test compared to the ELISA. The 2000-2002 Newfoundland survey showed better agreement between the tests. The ELISA indicated a higher prevalence of *A. vasorum* infection than the Baermann exam, but this difference was not statistically significant. The discrepancy in the comparison of the tests between the two surveys may indicate that lower infection intensities were present in the dogs in the first survey. If such were the case, the larval shedding levels may have been lower and more variable. This would have negatively affected the Baermann test sensitivity. Furthermore, both sandwich ELISA and fecal testing indicate a marked increase in detection of *A. vasorum* in the study population from 1998-2000 to 2000-2002.

Further work is needed to confirm the *A. vasorum* infection status of the Baermann negative/ELISA positive dogs. The sandwich ELISA cut-off value

determined from dogs with known infection status lead to an estimated specificity of 1. Although this value may be unrealistically high, it suggests that the Baermann negative/ELISA positive results most likely indicate infected dogs. The sandwich ELISA did not detect antigen in the sera of 2 of the 24 Baermann positive dogs naturally infected with *A. vasorum*. False negatives occur in the *Dirofilaria* antigen tests in cases where there are very low worm burdens (Nelson *et al.*, 2005). Low worm burdens may have played a role in the false negative results on these 2 dogs. At the present time, the number of *A. vasorum* present needed to produce a detectable level of antigenemia is unknown.

Currently, fecal diagnostics are not utilized until dogs show clinical signs suggestive of *A. vasorum* infection. The availability of an accurate, affordable diagnostic method and a move towards routine diagnostic screening in healthy dogs will allow earlier detection of *A. vasorum* infected animals. The treatment prognosis is greatly improved in *D. immitis* infected dogs treated prior to the onset of clinical disease compared to those already showing signs of heart failure. The same would presumably be true for *A. vasorum* dogs.

To standardize the test, to increase sensitivity and develop a dependable source of diagnostic antigen(s), we attempted to characterize the antigen(s) detected in the sandwich ELISA test using molecular biology techniques (Chapter 3). This project has major significance for veterinary medicine. Currently the parasite is restricted to Newfoundland. Given that infected dogs could shed larvae for prolonged periods of time, the abundance of gastropods and red foxes in the region, and the frequency of travel between Newfoundland and the Maritime provinces it seems inevitable that *A.*

vasorum will spread throughout Atlantic Canada. The use of the sandwich ELISA test shows promise as a tool for the diagnosis of this important pathogenic parasite of dogs. Accurate diagnosis is essential to the medical management of angiostrongylosis in dogs.

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Table 2.1: Sources of dog sera used to determine performance of the sandwich ELISA and in field evaluation of the test.

Source	*A. vasorum* positive#	*A. vasorum* negative#	Total
Non endemic	0	52	52[a]
Non endemic *C. vulpis* positive	0	30	30[a]
Newfoundland	24[b]	215[c]	239

by Baermann test
[a] Used as *A. vasorum* negative sera
[b] Used as *A. vasorum* positive sera
[c] Dogs from the endemic region with clinical signs consistent with *A. vasorum* infection

Table 2.2: Indirect ELISA antibody test, using *A. vasorum* antigen and tested with sera from 30 Baermann positive *C. vulpis* dogs from non endemic areas for *A. vasorum* (NB, PEI, NS) and 24 *A. vasorum* Baermann positive dogs from Newfoundland.

	***C. vulpis* positive dog sera**	***A. vasorum* positive dog sera**
Minimum OD value	0.240	0.255
Maximum OD value	0.530	0.569
Mean OD value	0.372	0.407
SD	0.076	0.093
Negative Controls [a]	0.003, 0.005	0.003, 0.005

[a] Sera from Minnesota that were Baermann and ELISA negative for both parasites.

Table 2.3: Optical densities from sandwich ELISA on sera from 3 Baermann positive dogs for *A. vasorum* using different combinations of polyclonal and monoclonal antibody.

Dog Sera	Polyclonal/ Monoclonal[a]	Monoclonal/ Polyclonal[b]	Monoclonal/ Monoclonal[c]	Polyclonal/ Polyclonal[d]
Dog # 1 serum	0.123	0.141	0.132	0.463
Dog # 2 serum	0.032	0.139	0.110	0.452
Dog # 3 serum	0.091	0.036	0.081	0.380
Negative serum	0.006	0.002	0.003	0.004

[a] Polyclonal affinity purified antibody was used as the capture antibody and monoclonal antibody affinity purified as the detecting antibody.
[b] Monoclonal antibody was used as the capture antibody and polyclonal as the detecting antibody
[c] Monoclonal antibody was used as the capture antibody and as the detecting antibody.
[d] Affinity purified polyclonal antibody was used as capture and affinity purified polyclonal antibody labelled with biotin was used as the detecting antibody.

Table 2.4: Determination of specificity and sensitivity of the ELISA test using dogs from Minnesota and AVC which were negative for both *C. vulpis* and *A. vasorum*, and dogs from Newfoundland which were *A. vasorum* Baermann positive.

	Baermann Positive	Baermann Negative	Total Number
ELISA Positive	22	0	22
ELISA Negative	2	52	54
Total Number	24	52	76

Table 2.5: Sandwich ELISA and Baermann fecal examination from dogs in Newfoundland suffering from cardiopulmonary disease (chronic cough) from two surveys, between the years 1998-2000 and 2000-2002.

	Baermann Positive	Baermann Negative	Total number
1998-2000			
ELISA Positive	5	17	22
ELISA Negative	0	160	160
Total number	5	177	182
2000-2002			
ELISA Positive	17	6	23
ELISA Negative	2	32	34
Total number	19	38	57

Table 2.6: Sandwich ELISA and parasite recovery from the experimental infection of 10 dogs with 150 third-stage larvae of *Angiostrongylus vasorum*.

Number of Dog	Total** Worm Count	Pre-Exposure	2 week PI OD*	4 week PI OD	6 week PI OD	8 week PI OD	10 week PI OD	11 week PI OD	Quantitative Baermann Examinations***				
									Week 10	Week 11	Week 12	Week 13	Week 14 Necropsy
1	67	0.09	0.19	0.24	0.32	0.33	0.33	0.32	5.0	42.0	93.8	219.1	507.7
2	88	0.09	0.27	0.27	0.37	0.38	0.37	0.36	280.9	104.7	24.0	874.7	3282.2
3	82	0.09	0.26	0.26	0.33	0.35	0.33	0.30	275.9	83.8	915.2	617.5	1137.7
4	59	0.03	0.20	0.22	0.35	0.33	0.33	0.29	451.4	2184.2	945.5	1284.4	5719
5	105	0.12	0.17	0.20	0.29	0.31	0.32	0.32	361.5	3168.0	356.1	1401.9	21098.5
6	56	0.06	0.23	0.23	0.31	0.30	0.32	0.30	59.5	1.0	15.0	63.2	446.5
7	41	0.09	0.17	0.21	0.37	0.37	0.34	0.32	26.0	50.9	46.9	18.9	1034.1
8	73	0.10	0.16	0.24	0.25	0.31	0.34	0.29	126.7	41.9	532.3	119.4	4775.7
9	99	ND	0.17	0.23	0.30	0.30	0.32	0.30	5.0	110.0	304.2	1864.1	1276.6
10	72	0.04	0.25	0.27	0.31	0.30	0.33	0.35	656.0	58.8	58.9	160.4	3985.8
MN	74.2	0.07	0.21	0.24	0.32	0.33	0.33	0.32	224.8	584.5	329.2	662.3	4326.4
SD	18.8	0.04	0.04	0.02	0.04	0.03	0.01	0.02	208.5	1069.1	341.4	627.8	5870.2

*OD- Optical density reading from the sandwich ELISA test
** Total worms recovered from necropsy
*** L1 larvae/g feces
ND- not done
PI- Post Infection

Figure 2.1: Titration of the crude *A. vasorum* antigen in the sandwich ELISA using different combinations of monoclonal antibody and polyclonal antibody.

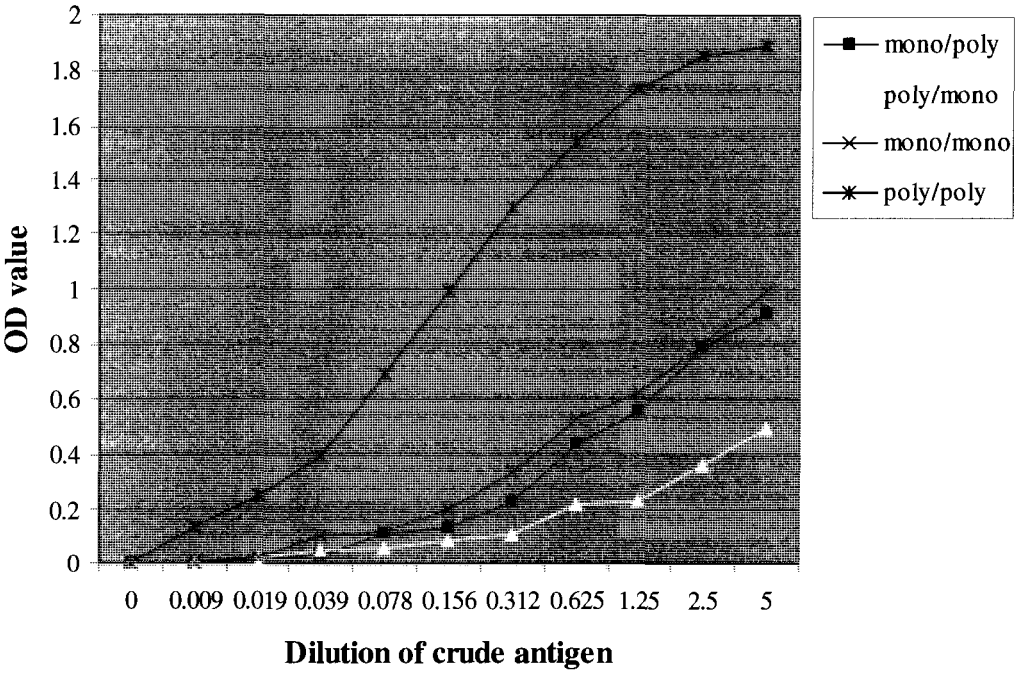


Figure 2.2: Two-graph receiver operating characteristic curve (TG-ROC) depicting sensitivity and specificity using ELISA optical densities from sera from dogs that were Baermann positive and negative for *A. vasorum*.

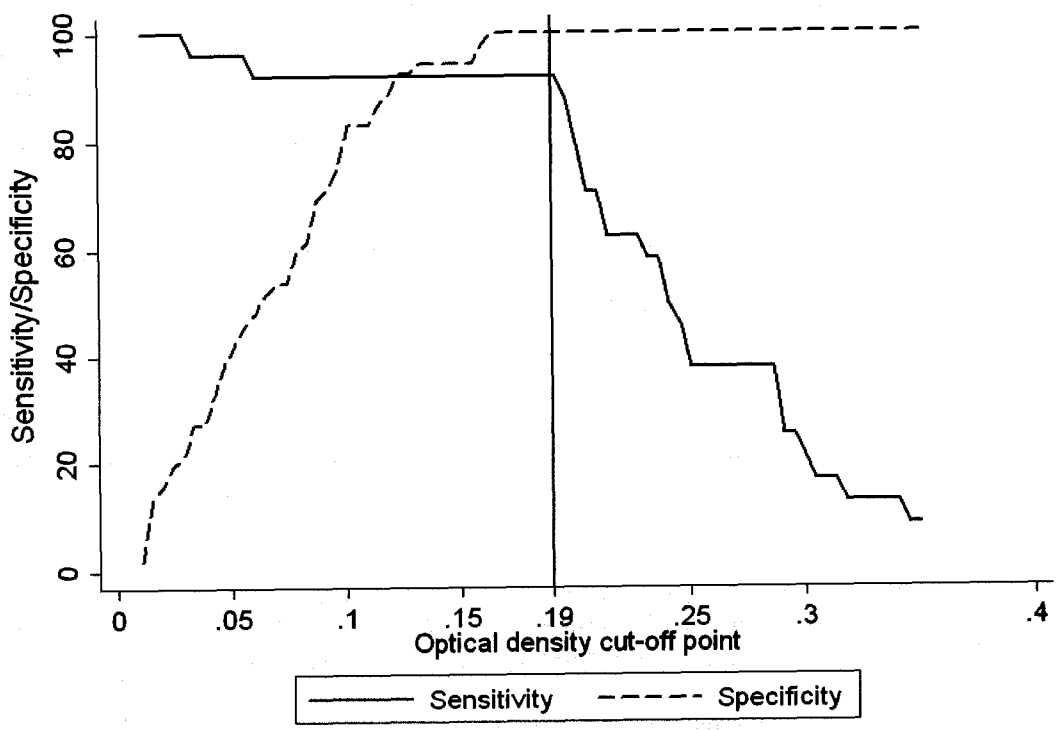
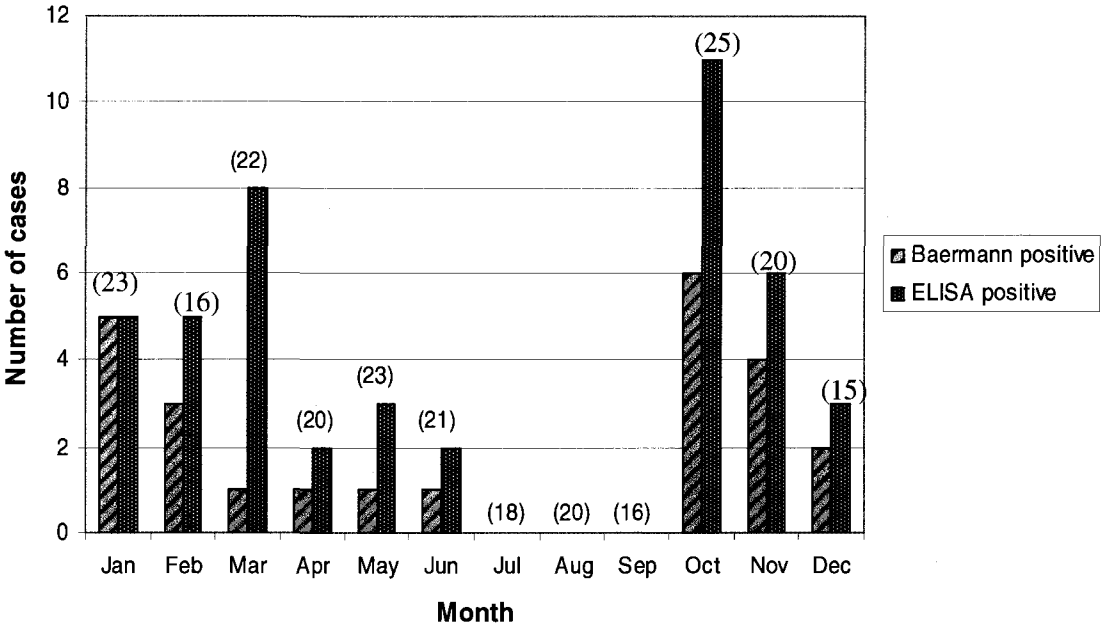


Figure 2.3: Sandwich ELISA and Baermann fecal examination from dogs with clinical signs of chronic cough, distributed by month of testing.



Note: total submission of cases for each month shown in parenthesis

CHAPTER 3: MOLECULAR IDENTIFICATION OF *A. VASORUM* BY SCREENING A cDNA EXPRESSION LIBRARY FOR IMMUNOREACTIVE CLONES

3.1 ABSTRACT

Angiostrongylus vasorum is a nematode parasite that infects the pulmonary artery and right ventricle of wild and domestic canids. Potentially fatal cardiopulmonary disease can occur in infected dogs. In North America, there is an endemic infection focus in Newfoundland, Canada. Diagnosis of infection in dogs occurs by detection of first-stage larvae by Baermann fecal examination. A sandwich ELISA based on rabbit polyclonal antiserum prepared against ground whole-nematode adult antigen was developed for the detection of circulating antigen in infected dogs. Test sensitivity/specificity was 92%/100%. To standardize the test, increase sensitivity, and develop a dependable source of diagnostic antigen(s), this study identified genes encoding relevant antigens. A cDNA library was constructed in the λZapExpress vector system that contain pBluescript SK- plasmid using poly (A) RNA purified from adult female and male *A. vasorum*. Screening of the amplified cDNA library using rabbit anti-*A. vasorum* serum revealed 8 immunoreactive clones. The sequence analysis showed these clones to contain sequences homologous to 3 proteins: vitellogenin, tropomyosin and heat shock 70 protein (Hsp70) which shared homology to similar proteins in other parasites. One unique immunoreactive clone from the *A. vasorum* cDNA library was also identified. These proteins most likely represent the principal antigens present in the whole-nematode adult antigen(s) that are being recognized by polyclonal serum in the sandwich ELISA.

3.2 INTRODUCTION

Angiostrongylus vasorum can cause potentially fatal cardiovascular disease in dogs and wild canids in various regions of the world. In North America it is restricted to the southeastern portion of Newfoundland, Canada. Diagnosis in dogs occurs by detection of first-stage larvae by Baermann fecal examination. A sandwich ELISA based on rabbit polyclonal antiserum prepared against ground whole-nematode adult antigen was developed for the detection of circulating antigen in infected dogs and was comparable or better than the Baermann test. Molecular characterization of the antigen(s) being detected in the sandwich ELISA will be necessary for generation of specific monoclonal antibodies which will increase the sensitivity of the sandwich ELISA test and generate consistent reagents for the test. Currently there is no genetic sequence information available for *Angiostrongylus vasorum.* To obtain relevant DNA sequence or at least partial sequences, a cDNA library was chosen as the most appropriate way to identify *A. vasorum* proteins. By cloning the cDNA into a vector capable of expressing *A. vasorum* proteins, immunoreactive clones can be identified by screening with polyclonal antiserum from rabbits immunized with whole *A. vasorum.* This would identify *A. vasorum* genes coding for immunoreactive proteins.

Several researchers have constructed cDNA libraries and used them to identify immunoreactive proteins from helminths. Vercauteren *et al.* (2002) identified secretory-excretory proteins of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. Donelson *et al.* (1988) reported construction of *Onchocerca volvulus* cDNA libraries. They had millions of clones from 4 cDNA libraries and randomly chose approximately 30,000 clones which they immunoscreened using human antisera

and rabbit polyclonal antisera and found only 4 positive clones. They found some similarities to paramyosin from other parasites and the authors suggested that further immunoscreening needed to be performed. Unnasch *et al.* (1988) also constructed an adult *Onchocerca volvulus* cDNA library, and had thousands of clones of which 6 reacted with rabbit polyclonal antisera. They did not find homology to published sequences held in the GenBank database. Construction of cDNA libraries from *Dictyocaulus viviparous* was reported by von Samson-Himmelstjerna *et al.* (1997); from thousands of clones they randomly selected 11 for screening. They found 9 sequences ranging from 250-700 base pairs (bp) and one in particular had similarities to the actin protein of the nematode *Caenorhabditis elegans.*

The current study focused on the development of a cDNA library using poly (A) RNA from adult female and male *A. vasorum* to identify immunoreactive proteins by screening clones with the same polyclonal antibody that was used in the sandwich ELISA test. In this study the Uni-ZAP XR vector lambda (λ) system that contains pBluescript was used for cDNA library expression. The pBluescript has bacteriophage f1 origin for replication as well as an *E. coli* origin of replication allowing isolation of phage and plasmid DNA which can be used for sequencing.

As little or no genetic information on *A. vasorum* is available, the first step was the identification of genes encoding immunoreactive proteins. By cloning genes of *A. vasorum* in an expression cDNA library and screening the clones for immunoreactivity, antigens can be identified and proteins can be produced which can be used for the generation of specific polyclonal and monoclonal antibodies. These antibodies can be then used in the sandwich ELISA to diagnose *A. vasorum* infection in dogs. In this

chapter the construction and use of such an expression cDNA library from adult *A. vasorum* is described. Several immunoreactive clones and their corresponding proteins were identified for the first time.

3.3 MATERIALS AND METHODS

3.3.1 RNA isolation

To avoid contamination with RNases, all tips, containers, tubes and pipettes were treated with DEPC (diethylpyrocarbonate). Three female and two male adult *A. vasorum* parasites were obtained from pulmonary arteries of infected dogs. The parasites were washed twice with saline containing 1% penicillin/streptomycin (Sigma) and then were soaked in saline for 30 min. One hundred and ten milligrams of the parasite were homogenized by grinding in liquid nitrogen with a mortar and pestle. After homogenization, 1.5 ml of Trizol (Invitrogen) was added and the parasites were homogenized again. The homogenate was transferred to a 15 ml sterile polystyrene tube and the mixture was incubated at room-temperature for 5 min. Following incubation, centrifugation was performed at 12,000 x g for 10 min at 4°C. Supernatant was transferred to a new microcentrifuge tube and 250 µl chloroform was added to the supernatant. The tube was shaken vigorously for 15 sec and incubated at room-temperature for 5 min. Centrifugation was performed at 12,000 x g at 4°C for 10 min. Again the supernatant was collected and transferred into a new tube and 600 µl of isopropanol (Fisher) was added. This was incubated at room-temperature for 10 min and was then centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1.1 ml 75% ethanol and incubated overnight

in a -80°C freezer. The RNA was precipitated in 15 µl of sodium acetate and with 413 µl (x 2.5 v/v ethanol) of 100% ethanol; the mixture was incubated overnight at -80°C. Following incubation, tubes were centrifuged for 30 min at 12,000 x g at 4°C. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellet and again centrifugation took place for 5 min at 12,000 x g at 4°C. The supernatant was again discarded and the pellet was dried for 8 min using vacuum centrifugation. When the pellet was completely dried, 150 µl of formamide (Fisher) was added. A portion of the RNA sample was then analysed using 1.5% agarose electrophoresis while the rest of the RNA was stored at -80°C in formamide for later use.

3.3.2 RNA electrophoresis

The quality of the isolated RNA was evaluated using 1.5% agarose gel electrophoresis. Five hundred milligrams of agarose was mixed with 36 ml DEPC-treated H_2O and heated in a microwave. In the fume hood 5 ml of 10 x MOPS buffer (MOPS [morpholinopropanesulphonic acid], EDTA and Na Acetate) and 9 ml of formaldehyde were added to the agarose. To 5 µl of the RNA sample, 2 µl of 10 x MOPS, 4 µl of formaldehyde and 10 µl of formamide were added. This mixture was incubated at 65°C for 5 min, cooled on ice for 5 min and centrifuged for 10 sec at 12,000 x g at 4°C. Following centrifugation, 4 µl of RNA loading buffer (95% deionized formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 5 mM EDTA, 0.025% SDS) was added to the RNA sample with 1/2 µl of ethidium bromide. Electrophoresis was carried out at 100 V for 2 h.

3.3.3 Poly (A) RNA purification

Messenger RNA (mRNA) was isolated from total RNA using a Poly (A) Quik mRNA isolation kit according to the manufacturer's specifications (Stratagene). Briefly, a sample of isolated RNA was heated at 65°C for 5 min and then placed on ice and a volume of 10 x sample buffer was added to give a final concentration of 1 x sample buffer. Affinity columns provided with the kit were used to extract and purify the mRNA by binding to the poly (A) tail of the mRNA. A 10 ml syringe was attached by the plunger to the column. The storage buffer was pushed slowly (one drop every second) out of the column and the syringe was removed from the column and 200 µl of high salt buffer wash was added and pushed slowly with the syringe and this was repeated 4 x. The RNA sample was applied to the column and pushed very slowly with the syringe and the sample was collected into a sterile DEPC- treated microcentrifuge tube. This was repeated and pushed very slowly from the column. A wash of 200 µl of high salt buffer was added to the syringe and pushed two drops every second, this was repeated twice. The column was washed again 3 x with 200 µl low salt buffer. Eventually, 200 µl elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was applied to the column and was passed very slowly through the affinity medium using the syringe. This was repeated 4 x and the eluates were collected into a sterile microcentrifuge tube. Purity of mRNA isolation was determined by RNA gel electrophoresis as described above. The mRNA was stored at -80°C in formamide (Fisher) for later use.

3.3.4 cDNA library construction

The cDNA library was constructed using a Zap-cDNA Gigapack III gold cloning kit (Stratagene), with modifications as described below.

3.3.4.1 cDNA first-strand synthesis

A microcentrifuge tube was placed on ice and the following were added: 5 µl of 10 x first-strand buffer, 3 µl of first-strand methyl nucleotide mixture, 2 µl of linker-primer, and 1 µl RNase Block Ribonuclease Inhibitor. The mRNA stored in formamide in -80°C was precipitated and dried as described above, and then the pellet was resuspended in 37.5 µl of DEPC treated water and added to the tube. The mixture was incubated (to allow the primer to anneal) for 10 min at room-temperature. Reverse Transcriptase (StrataScript) was added to the mixture (1.5 µl) and the final volume of the first-strand reaction mixture was adjusted to 50 µl water and the mixture was incubated for 1 h at 42°C.

3.3.4.2 cDNA second-strand synthesis

The first-strand synthesis mixture was placed on ice. The following components from the kit were then added: 20 µl 10 x second strand buffer, 6 µl second dNTP mixture, 114 µl sterile distilled DEPC treated water and 2 µl [α-^{32}P] dNTP. The following enzymes were added: 2 µl RNase H and 11 µl DNA polymerase I. The mixture was vortexed for 5 sec and centrifuged at 12,000 x g for 10 sec. The tube was incubated for 2.5 h at 16°C and then was placed immediately on ice.

3.3.4.3 Blunting the cDNA termini

The following was added from the kit to the second strand: 23 µl of blunting dNTP mix and 2 µl of cloned *Pfu* DNA polymerase. The tube was mixed gently, centrifuged at 12,000 x g and incubated for 30 min at 72°C. After the incubation, 200 µl (1:1) of phenol-chloroform (Fisher) was added at pH 7.5. The tube was mixed and centrifuged at 14,000 x g for 2 min at room-temperature. The supernatant which contained the cDNA was transferred to a new microcentrifuge tube after which 200 µl of chloroform was added to the cDNA (1:1) and the mixture was vortexed and centrifuged at 14,000 x g for 2 min at room-temperature. The cDNA supernatant was transferred to a new tube and the DNA was precipitated with 20 µl of 3 M sodium acetate and 400 µl of 100% (1:1) ethanol. The reaction was vortexed and incubated overnight at -20°C. The next day the tube was centrifuged for 1 h at 14,000 x g and the radioactive supernatant was discarded in the radioactive waste, and the pellet was very gently washed with 500 µl of 70% ethanol by centrifugation at 14,000 x g for 2 min at room-temperature. The radioactive supernatant was aspirated and the tube was dried for 8 min using vacuum centrifugation. The pellet was then resuspended with 9 µl of EcoRI adapters and incubated for 30 min at 4°C.

3.3.4.4 Ligating EcoRI adapters

The microcentrifuge tube containing the blunted cDNA and EcoRI adapters was put on ice and 1 µl of 10 x ligase buffer, 1 µl of 10 mM rATP and 1 µl of T4 DNA ligase were added (Stratagene). The reaction was centrifuged at 12,000 x g for 5 sec to ensure the mixture was at the bottom of the tube, and incubated overnight at 8°C.

3.3.4.5 Phosphorylating the EcoRI ends

The ligation was taken from 8°C and was incubated at 70°C for 30 min. Following incubation, the tube was centrifuged for 5 sec at 12,000 x g and incubated at room-temperature for 5 min. The following components were added; 1 µl of 10x ligase buffer, 2 µl of 10 mM rATP, 5 µl of sterile water and 2 µl of T4 polynucleotide kinase. The reaction was incubated for 30 min at 37°C and then was heat inactivated at 70°C for 30 min.

3.3.4.6 Digesting with XhoI

Following the 70°C incubation, the tube with the phosphorylated sample was centrifuged for 5 sec, and incubated at room-temperature for 5 min. The tube was put on ice and 28 µl of XhoI buffer supplement was added with 3 µl of XhoI (Stratagene). The reaction was incubated for 1.5 h at 37°C. Following incubation, 5 µl of 10 x STE buffer (Stratagene) and 125 µl of 100% ethanol were added. The sample was incubated overnight at -20°C.

3.3.4.7 Size fractionation

The sample was taken from -20°C and centrifuged at 14,000 x g at room-temperature for 1 h. The supernatant was discarded and the pellet was dried in the vacuum centrifugation for 8 min, and resuspended with 14 µl of 1 x STE buffer. Size fractionation was done according the manufacturer's guidelines (Zap-cDNA Gigapack III gold cloning kit, Stratagene). The size fractionation of the cDNA which contained

[α-^{32}P] dNTP was analyzed on an agarose gel, which was placed on an x-ray film cassette and exposed overnight at -80°C.

3.3.4.8 Ligating the cDNA insert into phage

For expression, a cDNA library λ phage vector (Uni-ZAP XR) which contained pBluescript SK- phagemid was used (Stratagene). The vector was double digested with EcoR I and Xho I. The cDNA and the vector were put on ice and the ligation reaction consisted of 1 µl of the cDNA, 1/2 µl of 10 x ligation buffers, 1/2 µl of 10 mM rATP, 1 µl of the pre-digested Uni-ZAP XR vector, 1 µl of distilled sterile water and 1/2 µl T4 DNA ligase (4 units). The ligation was performed overnight at 12°C.

3.3.4.9 Packaging

After ligation, 4 µl of ligation mixture was packed into infective phage particles using GigaPack III Gold packaging extract and then used to infect *E. coli* (Stratagene) using the manufacturer's suggested protocol. Briefly, 1 µl of ligation product prepared as described in Section 3.3.4.8, was mixed with one freshly thawed tube of 25 µl packaging extract (mixture of phage coat proteins and the phage DNA-processing enzymes, Stratagene) by gentle stirring with a pipet tip. The tube was immediately centrifuged for 3 sec at 14,000 x g and subsequently incubated for 2 h at room-temperature. After the incubation, 500 µl of SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM TrisHCl, 0.1% gelatine, pH 7.5) was added, followed by 20 µl of chloroform. The tube was centrifuged at 12,000 x g to sediment the debris for 10 sec and the

supernatant containing the cDNA library was transferred to a fresh tube and stored at 4°C until further use.

3.3.4.10 Amplifying the library

It is important to amplify the library to provide a large, stable quantity of high-titre stock. Amplification was carried out according the manufacturer's protocol (GigaPack III Gold Cloning Kit, Stratagene). Briefly, amplification was performed over 3 days. On the first day, 50 ml of *E. coli* strain XL1-Blue cells were grown in LB broth with supplements (for one liter of broth; 10 ml 1 M $MgSO_4$, 10 ml 20% maltose) at 30°C with shaking until OD_{600} of 0.8 was obtained. The next day, the XL1-Blue cells were centrifuged gently at 1000 x g for 10 min at 4°C. The cell pellet was resuspended in 25 ml of 10 mM $MgSO_4$. The cells were measured again and diluted to an OD_{600} of 0.5 in 10 mM $MgSO_4$. In a 15 ml polypropylene tube (4 tubes in total), 100 µl of the cDNA library was incubated with 600 µl of XL1-Blue cells at 37°C for 15 min to allow the phage to attach to the cells. Six and one half ml of NZY top agar (for one liter: 5 g NaCl, 2 g $MgSO_4$, 5 g yeast extract, 10 g NZ amine, pH 7.5, 0.7% agarose) melted and cooled to 48°C, was mixed with 100 µl phage infected bacteria, and was spread evenly onto fresh 150 mm NZY agar plates (4 plates). The plates were left for 10 min then inverted and incubated at 37°C for 7 h. The plaques were not larger than 2 mm in diameter. The plates were covered with 10 ml of SM buffer and incubated at 4°C overnight with gentle agitation to allow phage to diffuse into the buffer. On the third day the supernatant was pooled from each plate into a sterile 50 ml polypropylene tube. The plates were rinsed again with 2 ml of SM buffer and the supernatant was added to

the 50 ml tube. Chloroform was added to a final concentration of 5%, the solution was mixed well and incubated for 15 min at room-temperature. Centrifugation was performed at 500 x g for 5 min at 4°C. The supernatant was recovered and transferred into a sterile 50 ml polypropylene tube. Dimethyl sulfoxide (DMSO) was added to a final volume of 7% and the library was aliquoted and stored at -80°C.

3.3.5 Screening the λ Library

3.3.5.1 Infecting cells for expression

To infect host cells for fragment expression, 600 µl of XL-1 Blue cells (Stratagene) were incubated with 1 µl of several dilutions (undiluted, 1:10 to 1:64,000) of the phage suspension diluted with SM buffer for 15 min at 37°C. Top agar [NZ amine (casein hydrolysate), yeast extract, NaCl and $MgSO_4.7H_2O$ (NZY medium)] mixed with the bacterial phage suspension was poured over a 150 mm NZY Petri dish. The plates were incubated overnight at 37°C, followed by a further incubation at 4°C for 2 h.

3.3.5.2 Immuno-screening the library

Two nitrocellulose membranes were submersed in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 30 min at room-temperature. The membranes were air-dried on 3 MM Whatman paper, placed on the agar plates containing the phage plaques, and incubated for 3.5 h at 37°C. Following incubation, the membranes were marked in 3 places using Indian ink for orientation. The membranes were then put in a Zip-lock® plastic bag with 1% BSA in TBS blocking solution and were incubated for 1

h at room-temperature with gentle agitation. After incubation the membranes were washed 3 x with TTBS each time for 7 min. The membranes were then incubated with the same anti-*A. vasorum* rabbit polyclonal antibody that was used in the sandwich ELISA. To minimize cross-reactivity with the *E. coli* host cells, the antiserum was absorbed twice (each time for 3 h) with 1% (w/v) of acetone powder made from the *E. coli* host cell (XL-1 Blue). The same absorption procedure was performed on the anti-rabbit IgG enzyme conjugate. The membranes were incubated with the absorbed antiserum that was diluted to 1:4000 in 1% BSA in TTBS for 1.5 h at room-temperature. The membranes were washed again 3 x as before and incubated for 1 h at room-temperature with alkaline phosphatase labeled goat anti-rabbit conjugate diluted at 1:2000 in 1% BSA in TTBS. The membranes were washed 3 x with TTBS and 2 x with TBS and then developed by addition of the alkaline phosphatase (AP) substrate [AP buffer (AP colour development buffer mixed with ddH_2O), reagent A, reagent B, BIO-RAD], incubation for 7 min after which the reaction was stopped by addition of excess water. Positive signals on the membranes were correlated with plaques on the original plates using the orientation marks and positive plaques were excised from the plate. These were resuspended in 500 µl of SM buffer and 13 µl of chloroform, and stored at 4°C. The positive plaques were re-plated and screened again. Plaques giving positive reactions were resuspended in SM buffer as before and 7% DMSO was added; plaques were stored at -80°C. A sample was taken from each positive plaque for use as a template for a PCR reaction.

3.3.6 PCR reaction

PCR was performed on all immunoractive clones from the library using the standard T3 and T7 primers (Sigma).

3.3.6.1 PCR of immunoreactive clones

The vector primers used were: T3 (5' ATT AAC CCT CAC TAA AG 3') and T7 (5' AAT ACG ACT CAC TAT AG 3') (Sigma). A PCR reaction was performed in 100 µl reaction volumes as follows: 5 µl of template (positive plaque), 5 µl of T7 and T3 primers (stock solution of 10 pmol/µl), 2 µl of 0.5 M dNTPs (Invitrogen), 10 µl of 10x reaction buffer (Amersham) and 72.5 µl of ddH_2O. These components were mixed gently and 0.5 µl of Taq (2.5 units, Amersham) was added. The same reaction was performed with negative control using water as a template. The PCR reaction was performed in a Peltier Thermal Cycler. The following protocol was used: samples were held for 5 min at 95°C (initial denaturation) followed by 30 cycles of 94°C for 1 min (denaturation), 50°C for 1 min (annealing) and 72°C for 3 min (extension), then a final extension of 10 min at 72°C. The protocol was ended by keeping the samples at 4°C until the next morning. The PCR products were analysed using 1% agarose gel electrophoresis in 0.5 x TBS buffer for 1 h and 40 min at 120 V. The bands from the positive plaques were visualized by adding SYBR Safe stain (Invitrogen) to the samples prior to loading them onto the gel.

3.3.6.2 Purification of PCR immunoreactive clones and sequence determination

Purification of PCR products was performed by using a High Pure PCR product purification kit (Roche) with modification of the manufacturer's protocol as described below. All centrifugations were performed at room-temperature. Duplicate DNA bands from each positive phage were cut from the agarose gel using a sterile scalpel and placed in a sterile Eppendorf microcentrifuge tube to which 700 µl of binding buffer was added. The sample was vortexed for 30 sec and incubated for 10 min at 56°C with brief vortexing every 2 min during the incubation. When the gel was completely dissolved, 350 µl of isopropanol was added. A high pure filter tube (Roche) was inserted into one collection tube. The sample was transferred to the upper reservoir of the filter tube twice: the first time 500 µl were transferred and the sample was centrifuged at 13,000 x g for 40 sec, the supernatant was discarded and the remaining 550 µl of the sample was added and centrifuged as before. The sample was washed twice with 500 µl washing buffer at 13,000 x g for 1 min. The flow-through solution was discarded together with the collection tube, and 50 µl elution buffer was added to the filter tube which was connected to a new sterile microcentrifuge tube. The sample was centrifuged for 1 min at 13,000 x g. The purified DNA samples were sent to the Guelph Molecular Supercenter Laboratory Services Division, University of Guelph. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST), GeneBank database (Altschul *et al.*, 1990; Altschul *et al.*, 2005).

3.4 RESULTS

3.4.1 RNA isolation

Using Trizol, total RNA was successfully isolated and sufficiently purified for further use in mRNA isolation and generation of the cDNA library. In Figure 3.1 28S and 18S rRNA bands in the gel are clearly visible with no degradation, indicating that intact RNA was present. The ratio of the absorbance reading (OD 260/280) of the RNA sample was 2.0 indicating that pure RNA free of contaminating DNA had been prepared (Turner *et al.*, 2005).

3.4.2 cDNA library

The cDNA obtained was analyzed following the second strand synthesis. In this case an aliquot of the radioactive sample was electrophoresed on an agarose gel, which was then placed on an X-ray film in a cassette and exposed overnight at -80°C. The cDNA was visualized on the X-ray film as a smear indicating the variable length of cDNA molecules (Figure 3.2, lane 4). Following size fractionation and packaging, inserts from the cDNA library were amplified using PCR with T7 and T3 primers, and analysed using 1% agarose gel electrophoresis. The amplified cDNA library (from Section 3.3.4.10) was also analysed by the same procedure. Amplicons were visualized using SYBR Safe stain. In Figure 3.3 the amplified cDNA library and the cDNA library before the amplification are visible.

3.4.3 Identification and characterization of sequences from immunoreactive clones derived from *A. vasorum* cDNA library

After testing different dilutions of phage, 1:8000 dilution was determined optimal for plating and screening the library. One plate was plated and two duplicate membranes were screened. Eight plaques reacted with the rabbit antiserum to *A. vasorum*. Positive plaques have dark colour due to conversion of the substrate (Figure 3.4). The positive plaques were subcloned again and plated. All plaques from each subclone showed positive reaction with the antiserum. A PCR reaction was performed on each immunoreactive clone using the T3 and T7 primers. Insert sizes of the positive clones from the cDNA library ranged between 1600-2100 base pairs (bp) in size and lane 6 shows a negative control using water as a template (Figure 3.5, lanes 2-5 and lanes 7-10). The immunoreactive clones from the PCR reaction were purified and sent for sequencing to the Guelph Molecular Supercenter Laboratory Services Division, University of Guelph. Each of the sequences of the 8 clones that were generated from the Guelph Molecular Laboratory contained a sequence from pBluescript plasmid at the beginning of the insert (immunoreactive clone) and a long poly (A) tail. The individual sequences ranged in size from 1082-1717 bp (Figures 3.6, 3.10, 3.13 and 3.15). When the plasmid sequence and most of the poly (A) tail were removed, the inserts (immunoreactive clone) sequences ranged in size from 606-2065 bp (Figures 3.7, 3.9, 3.11, 3.14 and 3.16). Nucleotide and amino acid sequence data was analyzed using BLAST (Altschul *et al.*, 1990; Altschul *et al.*, 2005). Similarities were found for each clone.

Four *A. vasorum* immunoreactive clones had 76% similarity to the vitellogenin protein of the nematode *Caenorhabditis elegans* (Table 3.1). Three of these clones overlapped giving a total length of 2065 bp (Figures 3.7 and 3.8), the 4th immunoreactive clone stands by itself with 1617 bp (Figure 3.9). Tropomyosin was the second protein to be identified from the immunoreactive clones with 889 bp and had 97% similarity to the tropomyosin protein of *Onchocerca volvulus, Trichinella spiralis* and *Trichostrongylus colubiformis* (Table 3.1). Another protein that was identified from the immunoreactive clones was heat shock protein 70 (Hsp70). Two clones overlapped each other giving a total length of 1532 bp and had 95% similarity to the Hsp70 with the parasites *Parastrongyloides trichosuri* and *Wuchereria bancrofti* and the nematode *Caenorhabditis elegans* (Table 3.1, Figure 3.12). The last clone had a unique sequence that did not have any similarities to sequences in the GenBank. It was 606 bp long and is potentially a unique or novel protein in *A. vasorum* (Table 3.1).

3.5 DISCUSSION

Complementary DNA (cDNA) libraries are useful and powerful tools for uncovering genes of interest in organisms. This study focused on the identification of genes encoding *A. vasorum* proteins which may serve as antigens in immunoassays. As this involved eukaryotic DNA, the genes can be very long because of numerous introns. The advantage of using a cDNA library is that the introns are spliced out and the mRNA sequence can be used as a template to synthesize cDNA (via reverse transcriptase) to clone the preferred genes. A genomic library contains DNA fragments representing the entire genome of an organism including introns that do not function in coding for

proteins and is better used in prokaryotic DNA which is much shorter and mRNA is difficult to isolate (Fulton *et al.*, 1995). In this study we were using a eukaryotic organism and looking for immunoreactive proteins from *A. vasorum* gene sequence, thus a cDNA library was preferred. Others have used similar approaches to produce cDNA libraries for *Onchocerca volvulus* and *Ostertagia ostertagi* (Vercauteren *et al.*, 2002; Donelson *et al.*, 1988). Vercauteren *et al.* (2002) constructed a cDNA library from *O. ostertagi*, and identified 117 immunoreactive clones from which they isolated 41 relevant proteins.

The current study identified a total of 8 immunoreactive clones and using BLAST (Altschul *et al.*, 1990; Altschul *et al.*, 2005) identified 3 protein homologues encoded for the 8 cDNA library immunoreactive clones. Some of the clones overlapped each other, and one encoded a potentially unique protein. The proteins sequences identified are partial sequences and since the cDNA synthesis was primed using Oligo (dT), the sequences represent the 3' end of the mRNA (or the C-terminus of the proteins) and the 5' end remains unknown. This represents the first DNA sequence characterization that has been done on *Angiostrongylus vasorum*. The identification of these proteins was considered significant in this study as it will allow production of recombinant proteins and permit development of new reagents for diagnostics.

Among the proteins identified, one particularly appeared to be similar to those identified by Vercauteren *et al.* (2002) in *O. ostertagi*. In our study, 4 proteins had significant homology to the vitellogenin protein. Likewise in the Vercauteren study, 117 immunoreactive clones were identified yielding 41 relevant proteins of which 30

proteins had significant homology to vitellogenin. They too could not assemble the full length of this protein due to its large size.

Generally, vitellogenin is the precursor of a yolk protein, and in humans it is a female-specific protein that is present in the blood. Vitellogenin is expressed in the liver under regulation of estrogen during the egg formation process, and when it reaches a growing ovarian follicle via the blood stream, it contributes to the egg yolk formation (Sharrock, 1983). The primary site of yolk protein synthesis in the nematode, *Caenorhabditis elegans*, has been determined by Sharrock (1983). Yolk proteins in nematodes are synthesized in the intestine, secreted from the intestine into the body cavity, and taken up from the body cavity by the gonad to reach oocytes (Sharrock, 1983). These exogenously derived yolk protein precursors are synthesized in the liver of vertebrates (Tata, 1988), in the digestive gland of snails (Barre *et al.*, 1991), in the fat body and the follicle cells in insects (Brennan *et al.*, 1982; Postlethwait and Jowett, 1980), and in the gut epithelium of nematodes (Kimble and Sharrock, 1983). From the cDNA library in this study, the sequence of 4 *A. vasorum* immunoreactive clones had 76% similarity to vitellogenin protein when compared to the nematode *C. elegans*. Three of these sequences overlapped with 2065 bp, one of these sequence stands by itself with 1618 bp. As was described above, vitellogenin protein is synthesized in adult females and also in juvenile forms, with increased synthesis during maturation and sexual reproduction. Vitellogenin protein is essential for parasites, and as rabbit polyclonal antisera detected this protein in the *A. vasorum* cDNA library screening, it is potentially antigenic and appears to be released during infection. It will be advantageous to develop antibody against the vitellogenin protein for diagnostic

purposes. Sera from dogs infected with *A. vasorum* potentially can be identified using the sandwich ELISA test with polyclonal antibody or a monoclonal antibody generated against vitellogenin.

Tropomyosin was the second protein homologue to be identified from the cDNA library. The sequence consisted of 889 bp and when GenBank was searched using BLAST , this sequence had 97% similarity to tropomyosin protein from *Onchocerca volvulus*, *Trichinella spiralis* and *Trichostrongylus colubriformis*. Tropomyosin is a major actin-associated protein in eukaryotes. It is an essential regulator of contraction of the myoepithelial sheath during ovulation in the *C. elegans* reproductive system (Ono and Ono, 2004). Contraction of the ovarian muscle requires tropomyosin and troponin, which are generally major actin-linked regulators of contraction of striated muscle. This protein is the component of thin filaments in the sheath cells of the *C. elegans* ovary and is required for ovarian contraction during ovulation. It is found in adults and larval stages of parasites in their muscle tissue helping in regulating motility of nematodes (Ono and Ono, 2004). Furthermore, Mansir and Justine (1996) observed the presence of actin and tropomyosin in spermatids and spermatozoa of the *Heligmosomoides polygyrus* a nematode parasite of woodmice and other rodents. Perez-Perez *et al.* (2000) and Nakada *et al.* (2003) demonstrated that tropomyosin can act as a pan-allergen (an allergen widely distributed in the nature), and cause allergic reaction in humans ingesting invertebrates like shrimp (and other crustaceans) or even from marine nematodes like *Anisakis simplex* contaminating fish products. Tropomyosin from nematodes can be antigenic and the DNA sequence of this protein is highly conserved (Nakada *et al.*, 2003). Because this protein has an important role in muscle contraction,

it is considered to be an essential protein for all parasites in all stages, hence, its antigenic properties and its usefulness in immunodiagnostic tools could be assessed. In future studies, tropomyosin fusion proteins from *A. vasorum* could be produced and purified and used as candidates for immunodiagnostic tools.

Another protein homologue that was identified from the immunoreactive clones in the *A. vasorum* cDNA library was Hsp70. Two sequences overlapped each other with 1939 bp, and compared to the GenBank data had 95% similarity to Hsp70 from *Parastrongyloides trichosuri* and *Wuchereria bancrofti* parasites and the free-living nematode *Caenorhabditis elegans*. The Hsp70s are a family of ubiquitously expressed proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are important during host invasion by parasitic nematodes and help to protect the parasites from stress and temperature changes. Nematodes need to endure sudden and sharp changes in temperature due to their life cycle, for example, living in gastropods and then shifting to warm blooded mammals (Maresca and Carratu, 1992). Hsp70s are expressed at all developmental stages of parasites and possess parasitic-specific antigenicity. Hence, Hsp70 is potentially a good candidate for use in a diagnostic tool (Bannai *et al.*, 2003: Dobbin *et al.*, 2002). For example, mice were immunized with mitochondrial Hsp70 from *Trypanosoma congolense*. Specific antibodies were produced and the primary stage of *T. congolense* infection was detected in infected sera on a sandwich ELISA test (Bannai *et al.*, 2003). As a follow-up to our study, Hsp70 fusion proteins produced from *A. vasorum* immunoreactive clones could be used to generate polyclonal or monoclonal antibodies and used in our sandwich ELISA protocol.

The last immunoreactive clone that was found had a unique sequence that did not match any sequence in GenBank, and thus could be a unique or novel protein of *A. vasorum*. As more genes and proteins are identified, as the number of nematode gene sequences increase in GenBank, sequence similarities to this unique *A. vasorum* may be discovered. Protein expression and purification of these specific fragments of antigens and production of monoclonal antibodies targeting the specific antigen and using it in the sandwich ELISA may improve the test sensitivity and will benefit for quick diagnosis of *A. vasorum* infected dogs.

In conclusion, a cDNA library was constructed and screened with rabbit antiserum against *A. vasorum* whole crude antigen. Eight immunoreactive clones were identified and sequenced. Analysis revealed 3 proteins (vitellogenin, tropomyosin and Hsp70) and one potentially unique protein. This is the first study to characterize DNA sequence from *A. vasorum* cDNA library immunoreactive clones.

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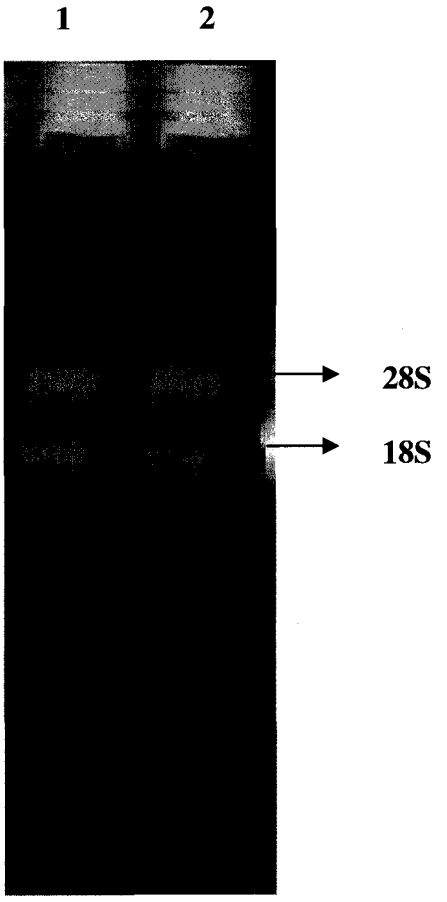
von Samson-Himmelstjerna., G., Wunderlich, G., Muhlschlegel, F., Frosch, M., Schnieder, T., 1997. Polymerase chain reaction-based construction of cDNA libraries from minute amounts of third-stage and fourth-and fifth-stage larvae of *Dictyocaulus viviparus*. Parasitology Research 83, 20-23.

Table 3.1: Summary of the immunoreactive clones from the *A. vasorum* cDNA library, their insert size with the corresponding accession numbers in the GenBank database, and greatest homology to other nematodes using the BLAST analysis.

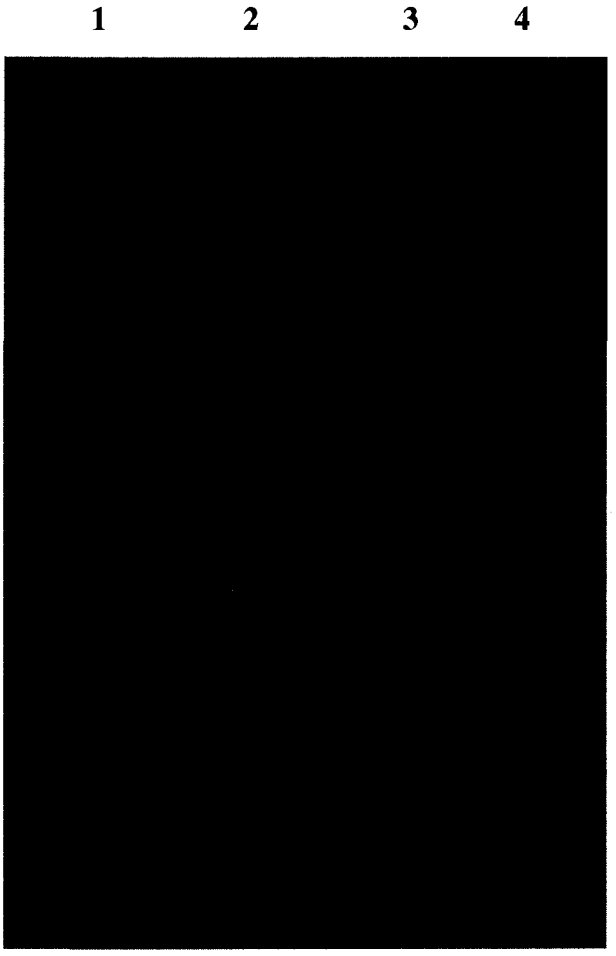
Clone #	Insert size in base pairs	Accession numbers	Homology to proteins in the GenBank database
1-2-3	2065	EF552219	Vitellogenin, 76% similarity to *Caenorhabditis elegans*
4	1618	EF552220	Vitellogenin protein, 76% similarity to *C. elegans*
5-6	1876	EF552221	Hsp70, 95% similarity to *C. elegans*, *Parastrongyloides trichosuri* *Wuchereria bancrofti*
7	889	EF552222	Tropomyosin, 97% similarity to *Onchocerca volvulus*, *Trichinella spiralis* *Trichostrongylus colubriformis*
8	606	EF552223	No similarity found

Figure 3.1: RNA agarose gel electrophoresis of total RNA extracted from *A. vasorum*.



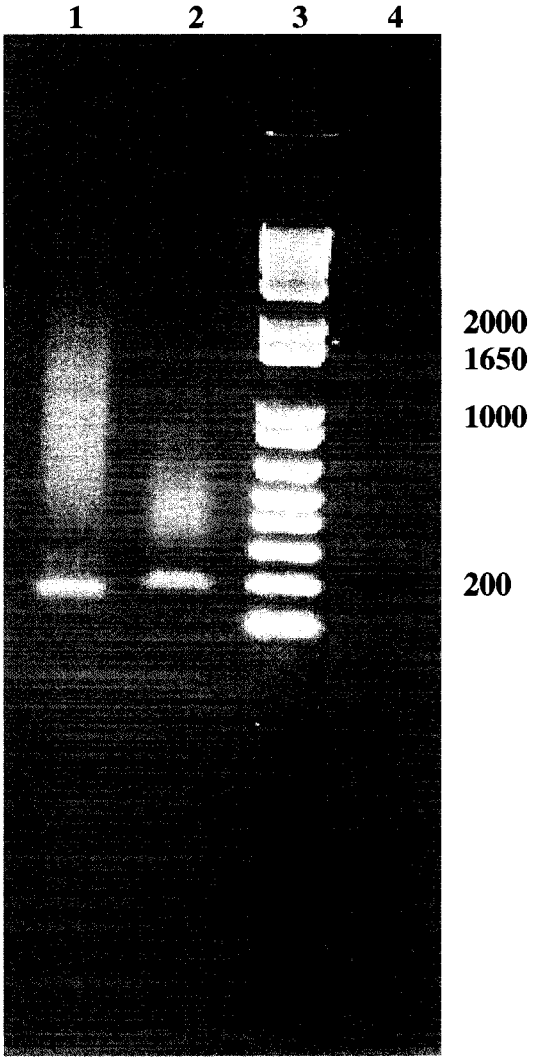
Note: **Lane 1 and 2**- Duplicate samples of total RNA showing two sharp bands, the upper 28S rRNA and the lower band is 18S rRNA

Figure 3.2: X-ray film showing the cDNA following second strand synthesis.



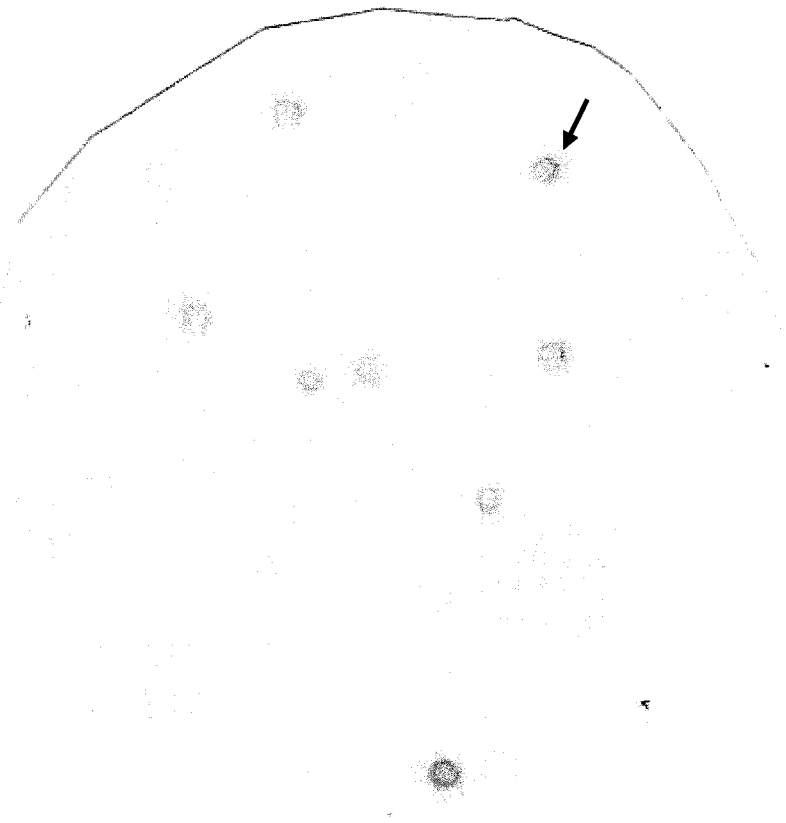
Note: **Lane 1 and 2**-1kB Plus DNA ladder, **lane 3**-empty (artefact is seen), **lane 4**-a smear is visible which indicates the variable length cDNA synthesis prodects

Figure 3.3: Agarose gel showing the PCR products derived from amplified and non amplified cDNA library of *A. vasorum*.



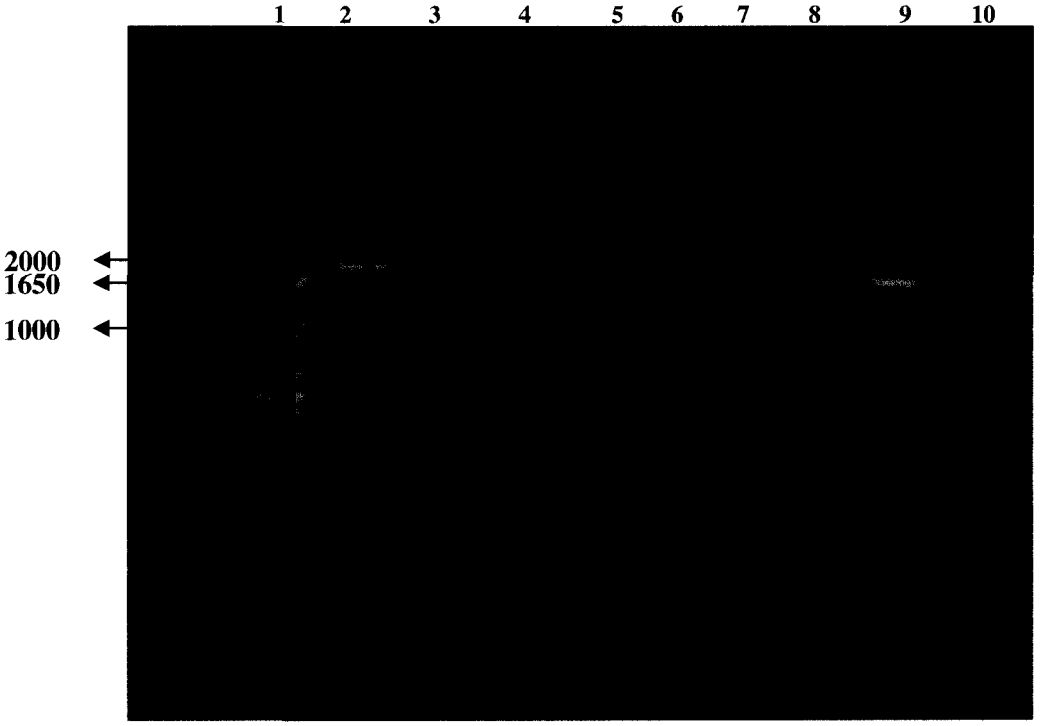
Note: **Lane 1**- amplified cDNA library, **Lane 2**- cDNA library following packaging but before the amplification protocol, **Lane** 3- 1 kb plus DNA ladder (Invitrogen). The small bands that are seen in lanes 1 and 2 are primers, **Lane 4**- Negative PCR control, using water as a template.

Figure 3.4: Immunoscreening of the *A. vasorum* cDNA library.



Note: The arrow indicate one the positive plaques with dark and intense colour; 8 immunoreactive clones are visible

Figure 3.5: PCR products generated from immunoreactive clones of the *A. vasorum* cDNA library, using T3 and T7 primers.



Note: **Lane 1**- 1 kb Plus DNA ladder (Invitrogen), **Lane 2**- Clone # 4, **Lane 3**- Clones # 1, **Lane 4**- Clone # 2, **Lane 5**- Clone # 3, **Lane 6-** negative PCR control, using water as a template, **Lane 7**- Clone # 5, **Lane 8-** Clone # 6, **Lane 9**- Clone # 7, **Lane 10**- Clone # 8

Figure 3.6: Sequences obtained from 4 immunoreactive clones from the A. vasorum cDNA library encoding partial sequence for a vitellogenin protein.

(bold nucleotide: these sequence data contain pBluescript plasmid sequence)

Clone # 1 (1641 bp)

CTGGAGCTCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGG
GGAGAACCTCCGCTACTGTAAGTTGACCGTCGAAGGAACCTTCTCGCCATCCGAAGACAAACGCCGTGATT
GGAAATTCTTCACTAGCATGCAAGTACTTTTGCCGAAAATGGCTAAATCCCTGACGGAGCTCAAAAATCAA
GCTCACCGCGAAATACAGGGATTAGTGCAGTCGAAATGGGGAGCCGACGAAATAAACGAGCTGAATGTGAA
GTTCCAAGTAGAGCAGAGCAAAGAGCAAAAAGAATGGAGAAAACTTGCTGACAAGGAGCACAACGGATTGA
CTGCTTACGACCTCCTTCGCCAAGCCTCGCTTCTCAACCAGATGAAGATCGTCGCAAACTACGATCTCACT
CCTTCCATGAAGAATTTCGTCCATCAACTTTACGATTACCTTAAAGGATACACTTTCTGGCACAACAAAGT
CACCACAAATAACGGTGAGCAAGGAAAAGTCTTCCTAAGGCTGAGTGTTGACCCCGTCAGTCGTAGTCTGC
TCAATGTGCTTCTGGAAACACCGCACGAACGCGTAGAAGTGAAAGATTTTGTTGTTCCTCAGCTCTACCTT
CCATCGATCGCCAAAAGATCGCTTCGTGACATCCGCGATGAGTTGATGAAAGAACAAGTCTGCGAAGTTAA
AAGCACCAAAGTCCGAACCTTTGATGACCTCGTTTTCCGCGCTCCGTTGACAAACTGCTTCTCCGTGATCG
CTAAGGACTGTTCAGAAGAGCCTCGTTTCGCCGTTCTTCTCAGAAAGATCCAGAAGAATGCTGATGAAAAA
GAGCTGAAAATTATCAATGAACGCCAAGAAGTCATCAAGGTAAGGATGGTTGATGGCAGACTTATGATTTT
CGTTGACAATGAGGAGGTTGACAGAGACAGCGTGCAAAACTACAACATCGAAAAGATAGAAGATAACATGA
TTCGTGTGAAATTAGAAGATCTCGAAGTTCGCTTCGACGGATATGATACCAAGGTCTACATTGGTAAACAC
ATGAGTCAAAGACAGTGCGGACTTTGTGGACACTTCGACGAAGATAAGGACAGCGAATTCTACACCCCGAA
CAAAGAGTACACCAGTGACGTTGAGGAGTTCCATAAGTCGTATCTTTTGACCGATAAGTGCGAAGTTGAGA
AGGAGTTCTCCTTTGAGAAGAAAGATTACGCAGTCGAAACGAATGAAGAACGCAGTGATGACTGGTTGAGC
ACCTATGATGATGACAATACTAGCAATGATTTGAAGGACTTTGACGAAGAACCGTTGAAAACCACCCATGT
AATGGAATTCCCACACCGCGTTTGCTTCTCGCTTGAACCCGTCCGTAAATGTCGCAAAAACGAGAAGATGG
ACGATATGGTCGAAAAGAAAGTCCGCTTCACCTGCCTTCCTCGCTCATCGCATGAGACGAGACAACTCCTT
CACAAAGCTCGCACCAGTGTTCTCGAGCTGAACGACTACCCTATTTCGTTCGTTGAGAACCTTCGAGTTCC
CCTGCATGCGTTGTCTACTAAACTTATGCGGCAACGAGACCGTTCAATAAAGCTTCTTAATACAAAAAAAA
AAAAAAAA

Clone # 2 (1403 bp)

TTCTGCGTCCATCGCATGACTTCCCTAAAAAACTACGCTTCTACCTGAACAGCGCAAGAATAGAGAAGGTG
CGTGTTGGCAACGTTGACTCCACCTACCTCCTTGACCAAAAGTTTGATGTTGAACCCAGCAAATATGAGAA
ATTCGAGGAGAGAACGCGCCGTGATCACATTAGTAAATTCGCTCGTGACATTGAGAGATCTGAAGGCTACA
AACACCACTTGAATCTGAGGGTTGAGGCTGTCGATTCGAACGTGGAGCGCAACGGAAACGCCAAAATCGTC
ACCGTTTGTGAGGAGAACCTCCGCTACTGTAAGTTGACCGTCGAAGGAACCTTCTCGCCATCCGAAGACAA
ACGCCGTGATTGGAAATTCTTCACTAGCATGCAAGTACTTTTGCCGAAAATGGCTAAATCCCTGACGGAGC
TCAAAAATCAAGCTCACCGCGAAATACAGGGATTAGTGCAGTCGAAATGGGGAGCCGACGAAATAAACGAG
CTGAATGTGAAGTTCCAAGTAGAGCAGAGCAAAGAGCAAAAAGAATGGAGAAAACTTGCTGACAAGGAGCA
CAACGGATTGACTGCTTACGACCTCCTTCGCCAAGCCTCGCTTCTCAACCAGATGAAGATCGTCGCAAACT
ACGATCTCACTCCTTCCATGAAGAATTTCGTCCATCAACTTTACGATTACCTTAAAGGATACACTTTCTGG
CACAACAAAGTCACCACAAATAACGGTGAGCAAGGAAAAGTCTTCCTAAGGCTGAGTGTTGACCCCGTCAG
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TGCGAAGTTAAAAGCACCAAAGTCCGAACCTTTGATGACCTCGTTTTCCGCGCTCCGTTGACAAACTGCTT
CTCCGTGATCGCTAAGGACTGTTCAGAAGAGCCTCGTTTCGCCGTTCTTCTCAGAAAGATCCAGAAGAATG
CTGATGAAAAAGAGCTGAAAATTATCAATGAACGCCAAGAAGTCATCAAGGTAAGGATGGTTGATGGCAGA
CTTATGATTTTCGTTGACAATGAGGAGGTTGACAGAGACAGCGTGCAAAACTACAACATCGAAAAGATAGA
AGATAACATGATTCGTGTGAAATTAGAAGATCTCGAAGTTCGCTTCGACGGATATGATACCAAGGTCTACA
TTGGTAAACACATGAGTCAAAGACAGTGCGGACTTTGTGGACACTTCGACGAAGATAAGGACAGCGAATTC
TACACCCCGAACAAGAGTACACCAGTGACGTTGAGGAGTTCCATAAGTCGTATC

Figure 3.6 (cont'd): Sequences obtained from 4 immunoreactive clones from the *A. vasorum* cDNA library encoding partial sequence for a vitellogenin protein.

(bold nucleotide: these sequence data contain pBluescript plasmid sequence)

Clone # 3 (1177 bp)

GTTCTTCCGTCTATTGTGTGGACCGAAATACTTTGCCGAACTTGAAGAGAAGTCCGTTGTTATTCCTAAGT
GGAGAAGAGTTGCCCATCAGAGCGAAAAGATGATCAATGTTTGGGGAATTAAAGCCATTATGCGAGGAAAC
TTCATCAATAACTGGGAGAAGCGCGACGTCCTTCTAGGAGATTACGACTGGGAGATCGTTCTGCGTCCATC
GCATGACTTCCCTAAAAAACTACGCTTCTACCTGAACAGCGCAAGAATAGAGAAGGTGCGTGTTGGCAACG
TTGACTCCACCTACCTCCTTGACCAAAAGTTTGATGTTGAACCCAGCAAATATGAGAAATTCGAGGAGAGA
ACGCGCCGTGATCACATTAGTAAATTCGCTCGTGACATTGATAGATCTGAAGGCTACAAACACCACTTGAA
TCTGAGGGTTGAGGCTGTCGATTCGAACGTGGAGCGCAACGGAAACGCCAAAATCGTCACCGTTTGTGAGG
AGAACCTCCGCTACTGTAAGTTGACCGTCGAAGGAACCTTCTCGCCATCCGAAGACAAACGCCGTGATTGG
AAATTCTTCACTAGCATGCAAGTACTTTTGCCGAAATGGCTAAATCCCTGACGGAGCTCAAAAATCAAGCT
CACCGCGAAATACAGGGATTAGTGCAGTCGAAATGGGGAGCCGACGAAATAAACGAGCTGAATGTGAAGTT
CCAAGTAGAGCAGAGCAAAGAGCAAAAAGAATGGAGAAAACTTGCTGACAAGGAGCACAACGGATTGACTG
CTTACGACCTCCTTCGCCAAGCCTCGCTTCTCAACCAGATGAAGATCGTCGCAAACTACGATCTCACTCCT
TCCATGAAGAATTTCGTCCATCAACTTTACGATTACCTTAAAGGATACACTTTCTGGCACAACAAAGTCAC
CACAAATAACGGTGAGCAAGGAAAAGTCTTCCTAAGGCTGAGTGTTGACCCCGTCAGTCGTAGTCTGCTCA
ATGTGCTTCTGGAAACACCGCACGAACGCGTAGAAGTGAAAGATTTTGTTGTTCCTCAGCTCTACCTTCCA
TCGATCGCCAAAAGATCGCTTCGTGACATCCGCGATGAGTTGATGAAAGAACAAGTCTGCGAAGTTAAAAG
CACCAAAGTCCCGAACCTTTGATGACCTCGTTTTCCGCGCT

Clone # 4 (1717 bp)

CTGGAGCTCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGCAG
GGTAATCAGTGACATGAAACCACGTACCATATCCAGCAAACACTTGGAACTTTTAGAGATGCCTGTTCAAT
TTGTCTACAAAAATGGAATGGTCGCTGAGCTGCAGTTCTCGGAAAAAGAAGAGGCATGGTCAGCGAACATA
AAGCGCTCCGTTATCAACATGCTTCAAATTAATCTTCACAAAGTCGGAAAAATCGATGAGACTAACGTAGA
TGGAGCATTGATGGTTCGCGAAAACGACTTCTTCAATGCTAACGAGAGAACTATCGAAGGGGACTGCGAAG
TGGCCTACACAGTTCTTAAGAAGAAAGATGACATCACTGAGGTTACCAAGTCAGTAAACTTCAATAAATGT
TCTCGCCGTCCACAGGCCAAATACAATTTCCGCTATTTATCTGAATGTCGCGACTGCAATGATGCCGACAA
CTTTGAGCCGAATACTGTCTATAACTATGTACTAGAGAACAACGGCTTGAAGAAAGTTGAGGTCATGAGTG
TGTACACCGTTACCATTGACAACCAACCTGTAATGAAGACCGAAGTCCGCGCTAGACTCAGCCTCGAAGAC
GTCAGAAAGATCAGACAGCAGTTCGAGTCATCCAGAGGCAAGACCGAAGGCCTAATTTATTCCAATGAGAT
GGAGAAACAGATTGAACGTTTCTATATGTATGGAGACGACACAGAGGTTCTTCCATATGAGCGTGTTACCG
ACAAAATTGAAGCCATTCATAAGATCATTGATGGGATCAGAGAGCCAAAGGAGAACAACTACGAAAACACT
GTCCTCGTGTCCCGTCTTGTTTCCATACTTCGTATGTGCTCTCTCCGCGAACTGAGTGCTGTCCACTCCGA
CATCTATATGAAGGGTGATGAGCGCCTAAGGGCTATAATGGAGTATTCGTTGGCCATCGCTGGTACGAAAA
ACACTGTTACTCACCTACTTCGCCACGTGCAGAAGGAAGATGTCAAGACCTCCAGAATTGTGGGCCTTCTC
AAGTCCATCCAAGAGCTGCCGTATCCTTCTTCCAAAGTCGTTGAAGAACTACTCCGCTTCGCTGATAGCGG
CGTTGTAAAACGTTCTCCAGCTGTCCATCAGACCACATGGCTTGCAATAGGATCAGTCATGCGCGGAGTAG
TTGGAGACACCATGGATGAGAACCTTTTGGTCGAGAATCACCGCGGACTGAAACAGAAATACCTCAACATG
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TGGAAGCGATCGACGCTCTCCGCCTGCTCAAGGATTCCATGCCTCGAAAACTGAAATCTGTTCTGATGCCT
GTTTACAAAAACCGTCTCGAACATCCTGAACTTAGAATGGCAGCTCTTGTCCGCATTATGCACACCCTACC
TCAACAATCTGTCGTCGCTCAGATAGTCTCCACCATGGAGCGTGATTCTAATCAGCAGGTAGCTGCCTTTA
CCTATGACTTGCTAAACTCGTTTGCAAAATCAACCCATAGCCTGCTACAAGAAACTCGCCAAAAAAAAAAA
AAAAAAAAAAAA

Figure 3.7: Overlapping sequences obtained from 3 immunoreactive clones from A. vasorum cDNA library encoding partial sequence for a vitellogenin protein.

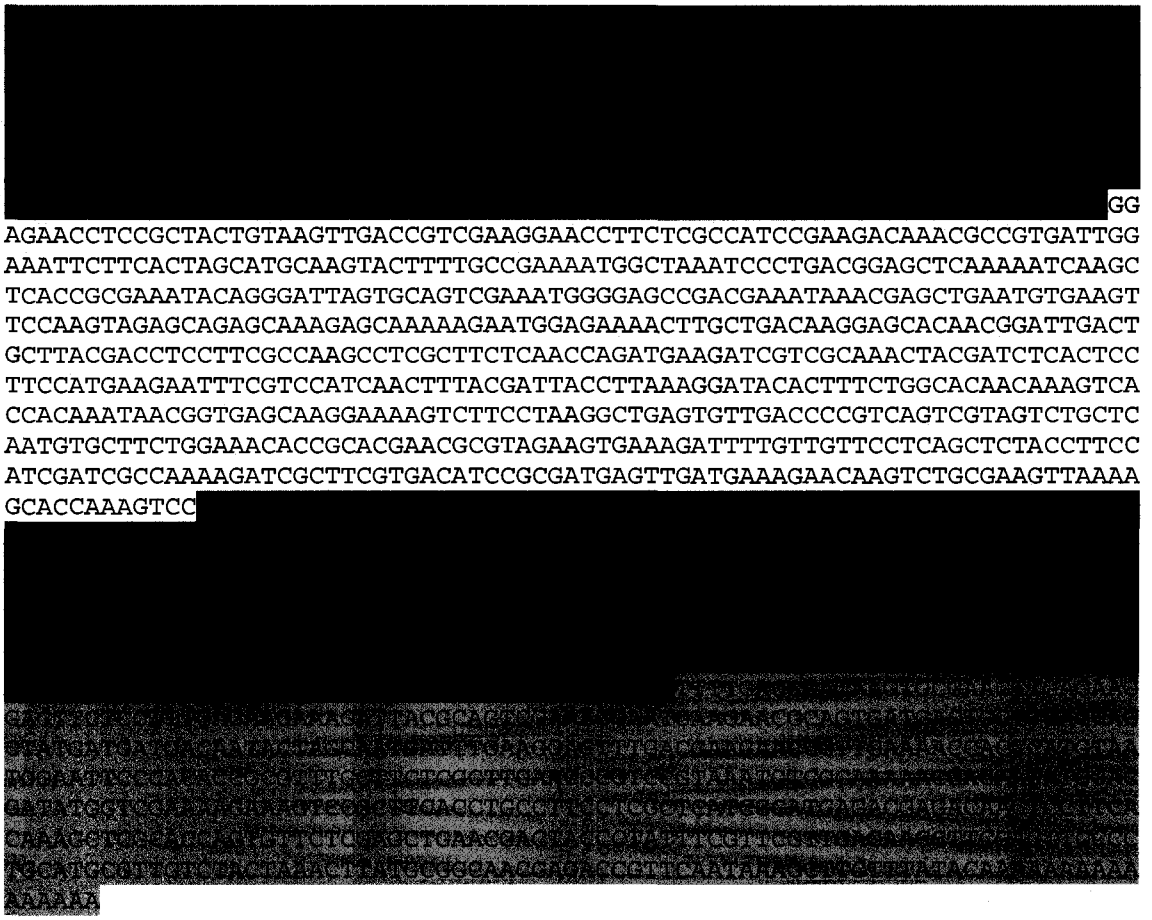
(these sequence data featuring the insert with pBluescript sequence and poly (A) removed)

Clones # 1-2-3 (2065bp)

```
1     GTTCTTCCGT CTATTGTGTG GACCGAAATA CTTTGCCGAA CTTGAAGAGA AGTCCGTTGT
61    TATTCCTAAG TGGAGAAGAG TTGCCCATCA GAGCGAAAAG ATGATCAATG TTTGGGGAAT
121   TAAAGCCATT ATGCGAGGAA ACTTCATCAA TAACTGGGAG AAGCGCGACG TCCTTCTAGG
181   AGATTACGAC TGGGAGATCG TTCTGCGTCC ATCGCATGAC TTCCCTAAAA AACTACGCTT
241   CTACCTGAAC AGCGCAAGAA TAGAGAAGGT GCGTGTTGGC AACGTTGACT CCACCTACCT
301   CCTTGACCAA AAGTTTGATG TTGAACCCAG CAAATATGAG AAATTCGAGG AGAGAACGCG
361   CCGTGATCAC ATTAGTAAAT TCGCTCGTGA CATTGAGAGA TCTGAAGGCT ACAAACACCA
421   CTTGAATCTG AGGGTTGAGG CTGTCGATTC GAACGTGGAG CGCAACGGAA ACGCCAAAAT
481   CGTCACCGTT TGTGAGGAGA ACCTCCGCTA CTGTAAGTTG ACCGTCGAAG GAACCTTCTC
541   GCCATCCGAA GACAAACGCC GTGATTGGAA ATTCTTCACT AGCATGCAAG TACTTTTGCC
601   GAAAATGGCT AAATCCCTGA CGGAGCTCAA AAATCAAGCT CACCGCGAAA TACAGGGATT
661   AGTGCAGTCG AAATGGGGAG CCGACGAAAT AAACGAGCTG AATGTGAAGT TCCAAGTAGA
721   GCAGAGCAAA GAGCAAAAAG AATGGAGAAA ACTTGCTGAC AAGGAGCACA ACGGATTGAC
781   TGCTTACGAC CTCCTTCGCC AAGCCTCGCT TCTCAACCAG ATGAAGATCG TCGCAAACTA
841   CGATCTCACT CCTTCCATGA AGAATTTCGT CCATCAACTT TACGATTACC TTAAAGGATA
901   CACTTTCTGG CACAACAAAG TCACCACAAA TAACGGTGAG CAAGGAAAAG TCTTCCTAAG
961   GCTGAGTGTT GACCCCGTCA GTCGTAGTCT GCTCAATGTG CTTCTGGAAA CACCGCACGA
1021  ACGCGTAGAA GTGAAAGATT TTGTTGTTCC TCAGCTCTAC CTTCCATCGA TCGCCAAAAG
1081  ATCGCTTCGT GACATCCGCG ATGAGTTGAT GAAAGAACAA GTCTGCGAAG TTAAAAGCAC
1141  CAAAGTCCGA ACCTTTGATG ACCTCGTTTT CCGCGCTCCG TTGACAAACT GCTTCTCCGT
1201  GATCGCTAAG GACTGTTCAG AAGAGCCTCG TTTCGCCGTT CTTCTCAGAA AGATCCAGAA
1261  GAATGCTGAT GAAAAAGAGC TGAAAATTAT CAATGAACGC CAAGAAGTCA TCAAGGTAAG
1321  GATGGTTGAT GGCAGACTTA TGATTTTCGT TGACAATGAG GAGGTTGACA GAGACAGCGT
1381  GCAAAACTAC AACATCGAAA AGATAGAAGA TAACATGATT CGTGTGAAAT TAGAAGATCT
1441  CGAAGTTCGC TTCGACGGAT ATGATACCAA GGTCTACATT GGTAAACACA TGAGTCAAAG
1501  ACAGTGCGGA CTTTGTGGAC ACTTCGACGA AGATAAGGAC AGCGAATTCT ACACCCCGAA
1561  CAAAGAGTAC ACCAGTGACG TTGAGGAGTT CCATAAGTCG TATCTTTTGA CCGATAAGTG
1621  CGAAGTTGAG AAGGAGTTCT CCTTTGAGAA GAAAGATTAC GCAGTCGAAA CGAATGAAGA
1681  ACGCAGTGAT GACTGGTTGA GCACCTATGA TGATGACAAT ACTAGCAATG ATTTGAAGGA
1741  CTTTGACGAA GAACCGTTGA AAACCACCCA TGTAATGGAA TTCCCACACC GCGTTTGCTT
1801  CTCGCTTGAA CCCGTCCGTA AATGTCGCAA AAACGAGAAG ATGGACGATA TGGTCGAAAA
1861  GAAAGTCCGC TTCACCTGCC TTCCTCGCTC ATCGCATGAG ACGAGACAAC TCCTTCACAA
1921  AGCTCGCACC AGTGTTCTCG AGCTGAACGA CTACCCTATT TCGTTCGTTG AGAACCTTCG
1981  AGTTCCCCTG CATGCGTTGT CTACTAAACT TATGCGGCAA CGAGACCGTT CAATAAAGCT
2041  TCTTTATACA AAAAAAAAAA AAAAA
```

Figure 3.8: Overlapping sequences of 3 immunoreactive clones from the A. vasorum cDNA library encoding partial sequence for a vitellogenin protein.

Clone # 1-2-3 overlapping sequence



Note: green- sequence unique to clone 3

red- sequence shared by clone 2 and 3

yellow- sequence shared by clone 1, 2 and 3

blue- sequence shared by clone 1 and 2

gray- sequence unique to clone 1

All clones extended to the poly (A) tail at the 3' end which was removed from clone # 3, 2 and part of the tail was removed from # 1.

Figure 3.9: Sequence obtained from one immunoreactive clone from the *A. vasorum* cDNA library encoding partial sequence for a vitellogenin protein.

(these sequence data featuring the insert with pBluescript sequence and poly (A) tail removed)

Clone # 4 (1618 bp)

```
1     ATCAGTGACA TGAAACCACG TACCATATCC AGCAAACACT TGGAACTTTT AGAGATGCCT
61    GTTCAATTTG TCTACAAAAA TGGAATGGTC GCTGAGCTGC AGTTCTCGGA AAAAGAAGAG
121   GCATGGTCAG CGAACATAAA GCGCTCCGTT ATCAACATGC TTCAAATTAA TCTTCACAAA
181   GTCGGAAAAA TCGATGAGAC TAACGTAGAT GGAGCATTGA TGGTTCGCGA AAACGACTTC
241   TTCAATGCTA ACGAGAGAAC TATCGAAGGG GACTGCGAAG TGGCCTACAC AGTTCTTAAG
301   AAGAAAGATG ACATCACTGA GGTTACCAAG TCAGTAAACT TCAATAAATG TTCTCGCCGT
361   CCACAGGCCA AATACAATTT CCGCTATTTA TCTGAATGTC GCGACTGCAA TGATGCCGAC
421   AACTTTGAGC CGAATACTGT CTATAACTAT GTACTAGAGA ACAACGGCTT GAAGAAAGTT
481   GAGGTCATGA GTGTGTACAC CGTTACCATT GACAACCAAC CTGTAATGAA GACCGAAGTC
541   CGCGCTAGAC TCAGCCTCGA AGACGTCAGA AAGATCAGAC AGCAGTTCGA GTCATCCAGA
601   GGCAAGACCG AAGGCCTAAT TTATTCCAAT GAGATGGAGA AACAGATTGA ACGTTTCTAT
661   ATGTATGGAG ACGACACAGA GGTTCTTCCA TATGAGCGTG TTACCGACAA AATTGAAGCC
721   ATTCATAAGA TCATTGATGG GATCAGAGAG CCAAAGGAGA ACAACTACGA AAACACTGTC
781   CTCGTGTCCC GTCTTGTTTC CATACTTCGT ATGTGCTCTC TCCGCGAACT GAGTGCTGTC
841   CACTCCGACA TCTATATGAA GGGTGATGAG CGCCTAAGGG CTATAATGGA GTATTCGTTG
901   GCCATCGCTG GTACGAAAAA CACTGTTACT CACCTACTTC GCCACGTGCA GAAGGAAGAT
961   GTCAAGACCT CCAGAATTGT GGGCCTTCTC AAGTCCATCC AAGAGCTGCC GTATCCTTCT
1021  TCCAAAGTCG TTGAAGAACT ACTCCGCTTC GCTGATAGCG GCGTTGTAAA ACGTTCTCCA
1081  GCTGTCCATC AGACCACATG GCTTGCAATA GGATCAGTCA TGCGCGGAGT AGTTGGAGAC
1141  ACCATGGATG AGAACCTTTT GGTCGAGAAT CACCGCGGAC TGAAACAGAA ATACCTCAAC
1201  ATGTTATTGA AGCAGTTTGA AAAAGCCGAC AAAATTTACG AAAAGGTGCT CGCTCTCAAG
1261  TCCCTCGCAA ACGCCGGAAT AGACCTATCT GTCCATGAAC TCGAAAAGAT TATCATGAAC
1321  AAGCATGAAG AGTTGCCTGT TCGTATGGAA GCGATCGACG CTCTCCGCCT GCTCAAGGAT
1381  TCCATGCCTC GAAAACTGAA ATCTGTTCTG ATGCCTGTTT ACAAAAACCG TCTCGAACAT
1441  CCTGAACTTA GAATGGCAGC TCTTGTCCGC ATTATGCACA CCCTACCTCA ACAATCTGTC
1501  GTCGCTCAGA TAGTCTCCAC CATGGAGCGT GATTCTAATC AGCAGGTAGC TGCCTTTACC
1561  TATGACTTGC TAAACTCGTT TGCAAAATCA ACCCATAGCC TGCTACAAGA AACTCGCC
```

Figure 3.10: Sequences obtained from 2 immunoreactive clones from the *A. vasorum* cDNA library encoding partial sequence for a Hsp70 protein.

(bold nucleotide: these sequence data contain pBluescript plasmid sequence)

Clone # 5 (1437 bp)

CTGGAGCTCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGCAG
GGTAAGTTCGACGACCCTGCAGTCCAGGCCGATATGAAGCATTGGCCGTTCAAAGTTGTTGCAGCCGAAGG
TTCCAAGCCCAAGGTACAGGTTGAATACAAAGGAGAGATCAAAGTGTTTACACCAGAAGAAATATCATCTA
TGGTTCTAACGAAGATGAAGGATACTGCCGAAGCCTTCCTGGGATCTACAGTCAAGGACGCCGTCATTACC
GTCCCCGCTTACTTTAATGACTCTCAGCGCCAAGCCACTAAGGATGCAGGAGCTATCGCGGGTCTTAATGT
CCTTCGAATCATAAACGAGCCAACAGCTGCTGCTATCGCCTATGGTCTCGACAAGAAGGGAACCGGGGAGA
GAAATGTCCTCATCTTTGATCTTGGAGGTGGAACCTTTGACGTGTCCATCCTGACCATTGAAGATGGCATT
TTCGAAGTGAAGTCTACTGCTGGAGACACGCATCTTGGAGGGGAGGACTTCGACAACCGCATGGTCAACCA
CTTTGTGGCAGAGTTCAAGCGAAAACACAAGAAAGACCTAAACACTAATCCTCGTGCGCTCAGGCGTCTTC
GTACTGCGTGCGAACGCGCAAAGCGTACTCTTTCATCGTCATCTCAGGCTTCCATTGAGATCGACTCACTC
TTCGAAGGCATCGATTTCTACACGAACATTACCCGTGCCCGGTTCGAGGAACTTTGTGCCGATCTCTTCCG
TTCGACCATGGACCTGTTGAGAAGTCTCTCAGAGATGCCAAGATGGACAAGAGTCAGGTGCAACGACATTG
TACTTGTTGGAGGCTCGACGCGTATCCCTAAAGTGCAAAAACTTCTATCTGATCTGTTCTCTGGGAAAGAA
CTGAACAAGTCCATCAACCCAGATGAAGCTGTCGCTTACGGTGCTGCTGTGCAGGCCGCCATCCTTTCAGG
CGATAAATCTGAAGCGGTCCAAGATCTTCTTCTTCTTGATGTCGCTCCACTCTCGCTTGGTATTGAGACTG
CTGGAGGTGTTATGACCGCTTTGATTAAGAGGAACACCACAATCCCTACAAAGACAAGCCAGACGTTTACG
ACATACTCCGACAACCAGCCTGGGGTATTGATCCAGGTCTTTGAAGGAGAACGAGCGATGACGAAAGACAA
TAACCTTCTTGGGAAGTTCGAGCTATCTGGAATTCCACCAGCTCCACGTGGAGTGCCACAGATTGAGGTTA
CCTTCGATATTGACGCTAATGGTATCTTAAATGTTTCTGCGCAGGACAAATCAACTGGAAAACAAAATAAG
ATTACTATCACGAACGACAAGGGACGGTTGTCGAAGGATGAGATTGAGCGTATGGTTAATGAAGCTGAGAA
ATACAAAGCGGAGGATG

Clone # 6 (1138 bp)

CTGGAGCTCCCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGG
CGTATCCCTAAAGTGCAAAAACTTCTATCTGATCTGTTCTCTGGGAAAGAACTGAACAAGTCCATCAACCC
AGATGAAGCTGTCGCTTACGGTGCTGCTGTGCAGGCCGCCATCCTTTCAGGCGATAAATCTGAAGCGGTCC
AAGATCTTCTTCTTCTTGATGTCGCTCCACTCTCGCTTGGTATTGAGACTGCTGGAGGTGTTATGACCGCT
TTGATTAAGAGGAACACCACAATCCCTACAAAGACAAGCCAGACGTTTACGACATACTCCGACAACCAGCC
TGGGGTATTGATCCAGGTCTTTGAAGGAGAACGAGCGATGACGAAAGACAATAACCTTCTTGGGAAGTTCG
AGCTATCTGGAATTCCACCAGCTCCACGTGGAGTGCCACAGATTGAGGTTACCTTCGATATTGACGCTAAT
GGTATCTTAAATGTTTCTGCGCAGGACAAATCAACTGGAAAACAAAATAAGATTACTATCACGAACGACAA
GGGACGGTTGTCGAAGGATGAGATTGAGCGTATGGTTAATGAAGCTGAGAAATACAAAGCGGAGGATGAAG
CACAGAAAGATCGTATTGGTGCGAAGAACTCTCTCGAAAGCTACGCTTTCAATATGAAGCAGACCCTCGAT
GACGAGAAGTTAAAGGACAAGATCTCAGCCGACGACCGTAAGAAAATTGAGAGCAAGTGTGATGAAGTCAT
CAGGTGGCTTTGACAGCAACCAGACCGCTGAGAAGGACGAGTTTGAACATCAGCAGAAGGAATTGGAAGCC
GTTTGCAACCCCATCATCACGAAAATGTACCAAAGTGCAGGAGGTGCACCCGGTGGTATGCCGGGCGGCAT
GCCTGGAGGAATGCCCGGAGGAATGCCAGGAGGTGGTTCTGCTGGCGGGCCCACCATTGAGGAAGTTGACT
GAGTCGTGTTCCGTAGTGATCTCCGAAATCTCAAGTCAAAAGTGTGGGTGGGCTAGTTCGACGCTCTCGTT
TATTTAGTTTTGTAACACTTTTGAATAATAAATATCTTTGTTGTTGAAAAAAAAAAAAAAAAAAAAAAAC
CC

Figure 3.11: Overlapping sequences obtained from 2 immunoreactive clones from the *A. vasorum* cDNA library encoding partial sequence for a Hsp70 protein.

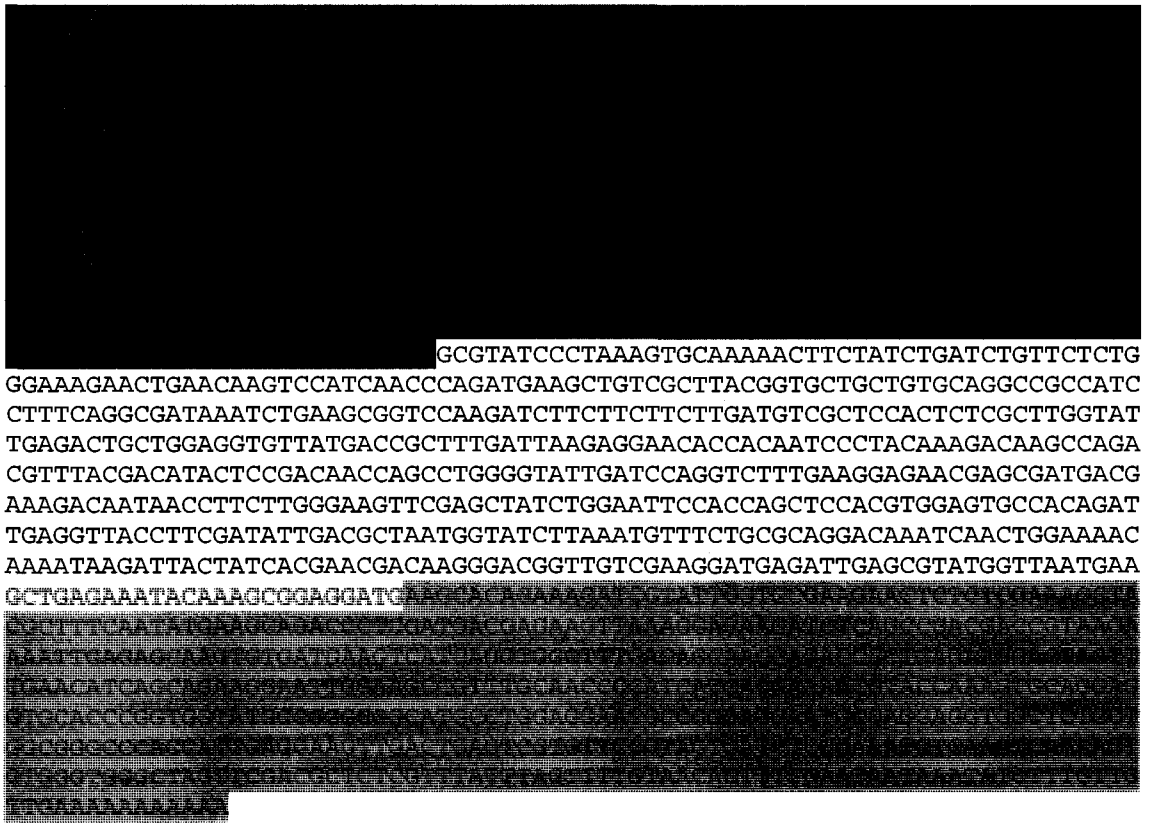
(these sequence data featuring the insert with pBluescript plasmid and poly (A) tail removed)

Clones # 5-6 (1876 bp)

```
1     GCACGCAGGG TAAGTTCGAC GACCCTGCAG TCCAGGCCGA TATGAAGCAT TGGCCGTTCA
61    AAGTTGTTGC AGCCGAAGGT TCCAAGCCCA AGGTACAGGT TGAATACAAA GGAGAGATCA
121   AAGTGTTTAC ACCAGAAGAA ATATCATCTA TGGTTCTAAC GAAGATGAAG GATACTGCCG
181   AAGCCTTCCT GGGATCTACA GTCAAGGACG CCGTCATTAC CGTCCCCGCT TACTTTAATG
241   ACTCTCAGCG CCAAGCCACT AAGGATGCAG GAGCTATCGC GGGTCTTAAT GTCCTTCGAA
301   TCATAAACGA GCCAACAGCT GCTGCTATCG CCTATGGTCT CGACAAGAAG GGAACCGGGG
361   AGAGAAATGT CCTCATCTTT GATCTTGGAG GTGGAACCTT TGACGTGTCC ATCCTGACCA
421   TTGAAGATGG CATTTTCGAA GTGAAGTCTA CTGCTGGAGA CACGCATCTT GGAGGGGAGG
481   ACTTCGACAA CCGCATGGTC AACCACTTTG TGGCAGAGTT CAAGCGAAAA CACAAGAAAG
541   ACCTAAACAC TAATCCTCGT GCGCTCAGGC GTCTTCGTAC TGCGTGCGAA CGCGCAAAGC
601   GTACTCTTTC ATCGTCATCT CAGGCTTCCA TTGAGATCGA CTCACTCTTC GAAGGCATCG
661   ATTTCTACAC GAACATTACC CGTGCCCGGT TCGAGGAACT TTGTGCCGAT CTCTTCCGTT
721   CGACCATGGA CCTGTTGAGA AGTCTCTCAG AGATGCCAAG ATGGACAAGA GTCAGGTGCA
781   ACGACATTGT ACTTGTTGGA GGCTCGACGC GTATCCCTAA AGTGCAAAAA CTTCTATCTG
841   ATCTGTTCTC TGGGAAAGAA CTGAACAAGT CCATCAACCC AGATGAAGCT GTCGCTTACG
901   GTGCTGCTGT GCAGGCCGCC ATCCTTTCAG GCGATAAATC TGAAGCGGTC CAAGATCTTC
961   TTCTTCTTGA TGTCGCTCCA CTCTCGCTTG GTATTGAGAC TGCTGGAGGT GTTATGACCG
1021  CTTTGATTAA GAGGAACACC ACAATCCCTA CAAAGACAAG CCAGACGTTT ACGACATACT
1081  CCGACAACCA GCCTGGGGTA TTGATCCAGG TCTTTGAAGG AGAACGAGCG ATGACGAAAG
1141  ACAATAACCT TCTTGGGAAG TTCGAGCTAT CTGGAATTCC ACCAGCTCCA CGTGGAGTGC
1201  CACAGATTGA GGTTACCTTC GATATTGACG CTAATGGTAT CTTAAATGTT TCTGCGCAGG
1261  ACAAATCAAC TGGAAAACAA AATAAGATTA CTATCACGAA CGACAAGGGA CGGTTGTCGA
1321  AGGATGAGAT TGAGCGTATG GTTAATGAAG CTGAGAAATA CAAAGCGGAG GATGAAGCAC
1381  AGAAAGATCG TATTGGTGCG AAGAACTCTC TCGAAAGCTA CGCTTTCAAT ATGAAGCAGA
1441  CCCTCGATGA CGAGAAGTTA AAGGACAAGA TCTCAGCCGA CGACCGTAAG AAAATTGAGA
1501  GCAAGTGTGA TGAAGTCATC AGGTGGCTTT GACAGCAACC AGACCGCTGA GAAGGACGAG
1561  TTTGAACATC AGCAGAAGGA ATTGGAAGCC GTTTGCAACC CCATCATCAC GAAAATGTAC
1621  CAAAGTGCAG GAGGTGCACC CGGTGGTATG CCGGGCGGCA TGCCTGGAGG AATGCCCGGA
1681  GGAATGCCAG GAGGTGGTTC TGCTGGCGGG CCCACCATTG AGGAAGTTGA CTGAGTCGTG
1741  TTCCGTAGTG ATCTCCGAAA TCTCAAGTCA AAAGTGTGGG TGGGCTAGTT CGACGCTCTC
1801  GTTTATTTAG TTTTGTAACA CTTTTGAATA ATAAATATCT TTGTTGTTGA AAAAAAAAAA
```

Figure 3.12: Overlapping sequences of immunoreactive clones from the *A. vasorum* cDNA library encoding partial sequence for a Hsp70 protein.

Clone #5-6 overlapping sequence



Note: red- sequence unique to clone 5
yellow- sequence shared by clone 5 and 6
grey- sequence unique to clone 6

All clones extended to the poly (A) tail at the 3' end which was removed from clone # 5 and part of the tail was removed from # 6

Figure 3.13: Sequences obtained from one immunoreactive clone from the *A. vasorum* cDNA library encoding partial sequence for a tropomyosin protein.

(bold nucleotide: these sequence data contain pBluescript plasmid sequence)

Clone # 7 (1242 bp)

CTGGAGCTCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGG
GGCTGCACTCCATTCCCTCTACAAACCACTAAGCAAAATGGACGCGATCAAGAAGAAGATGCAGGCGATGA
AGATCGAGAAGGACAACGCTCTCGATCGAGCCGATGCCGCCGAAGAGAAAGTCCGCCAGATTACCGAAAAG
TTGGAGCGGGTCGAGGAAGAACTTCGGGATACCCAGAAGAAGATGATGCAGACCGAGAACGACCTCGACAA
GGCGCAGGAAGATTTGGCCACTGCCACCAGCCAGTTGGAAGAGAAGGAGAAGAAGGTCCAAGAGGCTGAGG
CAGAAGTAGCTGCCTTGAATCGTCGCATGACACTGCTCGAAGAGGAGCTCGAGCGTGCTGAAGAACGCCTC
AAGATTGCTACCGAAAAACTCGAGGAAGCAACGCAGAACGTCGACGAGTCAGAGCGTGTACGCAAAGTGAT
GGAGAATCGTTCATTCCAAGATGAAGAGCGTGCAAATACGATTGAAGCTCAACTGAAGGAAGCACAGATGC
TTGCCGAGGAGGCCGATAGAAAATATGATGAGGTTGCCCGTAAGTTAGCAATGGTTGAAGCTGATCTCGAG
AGAGCAGAAGAACGTGCCGAAGCTGGAGAGAACAAAATCGTCGAATTGGAAGAAGAATTGCGTGTGGTTGG
TAACAACTTGAAGTCGCTCGAACTTTCCGAGGAAAAAGCTCTCGAGAAGGAAGACATATTCGCCGAACAGA
TTCCCCAACTTGACTACAGATTGAAAGAGGCTGAAACCCGTGCCGAGTTTGCTGAACGCTCCGTCCAAAAA
CTCCAGAAGGAAGTGGACAGACTCGAAGATGAACTGGTACACGAGAAGGAGAGATACAAAGCAATTTCGGA
GGAGCTTGATTCCACCTTCCAAGAGCTCTCCGGTTATTGATCCCTTCTTCCGCCACCGCTTCCTCATATTT
CACATTATTTCATTTCCTTCATACCTTTCCAGCATACCACTATTCACATTGTAGTCGGATAATTGTTCATT
CAATTCTGGCACGTCTGAACATGTTATTAAGCCTTGTCTTTATCGCTATTGCTTTTCGCAGTTCTTAGAGA
AGCAATTCACTCGACAGTTTTACTCTTAACATCAAATGTTTTTCTTATATTTTTATGCAGAGCAGGTCGAA
TAAAGAAATGAACGAAAAAAAAAAAAAAAAAGGGG

Figure 3.14: Sequences obtained from immunoreactive clones from the *A. vasorum* cDNA library encoding partial sequence for a tropomyosin protein.

(these sequence data featuring the insert with pBluescript sequence and the poly (A) tail removed)

Clone # 7 (889bp)

```
1     GGCTGCACTC CATTCCCTCT ACAAACCACT AAGCAAAATG GACGCGATCA AGAAGAAGAT
61    GCAGGCGATG AAGATCGAGA AGGACAACGC TCTCGATCGA GCCGATGCCG CCGAAGAGAA
121   AGTCCGCCAG ATTACCGAAA AGTTGGAGCG GGTCGAGGAA GAACTTCGGG ATACCCAGAA
181   GAAGATGATG CAGACCGAGA ACGACCTCGA CAAGGCGCAG GAAGATTTGG CCACTGCCAC
241   CAGCCAGTTG GAAGAGAAGG AGAAGAAGGT CCAAGAGGCT GAGGCAGAAG TAGCTGCCTT
301   GAATCGTCGC ATGACACTGC TCGAAGAGGA GCTCGAGCGT GCTGAAGAAC GCCTCAAGAT
361   TGCTACCGAA AAACTCGAGG AAGCAACGCA GAACGTCGAC GAGTCAGAGC GTGTACGCAA
421   AGTGATGGAG AATCGTTCAT TCCAAGATGA AGAGCGTGCA AATACGATTG AAGCTCAACT
481   GAAGGAAGCA CAGATGCTTG CCGAGGAGGC CGATAGAAAA TATGATGAGG TTGCCCGTAA
541   GTTAGCAATG GTTGAAGCTG ATCTCGAGAG AGCAGAAGAA CGTGCCGAAG CTGGAGAGAA
601   CAAAATCGTC GAATTGGAAG AAGAATTGCG TGTGGTTGGT AACAACTTGA AGTCGCTCGA
661   ACTTTCCGAG GAAAAAGCTC TCGAGAAGGA AGACATATTC GCCGAACAGA TTCCCCAACT
721   TGACTACAGA TTGAAAGAGG CTGAAACCCG TGCCGAGTTT GCTGAACGCT CCGTCCAAAA
781   ACTCCAGAAG GAAGTGGACA GACTCGAAGA TGAACTGGTA CACGAGAAGG AGAGATACAA
841   AGCAATTTCG GAGGAGCTTG ATTCCACCTT CCAAGAGCTC TCCGGTTAT
```

Figure 3.15: Sequences obtained from one immunoreactive clones from the *A. vasorum* cDNA library encoding for a unique protein.

(bold nucleotide: these sequence data contain pBluescript plasmid sequence)

Clone # 8 (1082 bp)

CTGGAGCTCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGG
TGGTGCTTCGCCTAATGGTAAATTTTGTGAAAGAAAAACATCTATGGATATCCCTGGCGTAGATGGTGTAC
ATAATCGACAAAACTGGTTTAATCAGCAAAGATGGTGGCTATTCATAAAACAGGCTGTATCGCTGCTTCTG
TCCGAGAATATGTTAAAAATACACCTCTTCATCTACTGGATCATTTGTCTGCTAACTACGATGGAAGACCG
ACAAAGTGTAAATACGTTAGCTGTTATGGTTATCGCAATGTGTATTGTGTTTTGCATCGTCGCACTGGTGA
CAAGAAATGCCTGTTTTTTCTACCCGTTTCTTTTTCTGGTGACTTTTGAATTTATGACGTCGTTGGCCCTA
TTTTGCCTTATAGCGTTAAAAATTGGATGGCCTCAAAACTATGTGGCTTTTGTAAAGGAAAATGAATTGGA
TTCTCTGCAGTATTTCGCGAAGTTCACTCATGAAACAAGTGAGGTGTCATGCTTGTGGGCTGGATTTCGAG
CTTTGTATTTTGCCCTAGTTGCTTTGACACTTGTAAAAAACATCACCGATTACCAAGTCGACGCGCATCAT
CGTCAACACAATCGCTACATTTATCCGCCGGTTCCTCCATATCCACGTCTTGTCGCGATACCTGAAACCCG
TAATGGGTAACACGAACCCTGATGATCCACCTCCGTATTCAGCGATAATTCGAGAACTAGCAGACTCCGGA
GAAACTGCAAAAGAAGAAACGCTTCCGCCTTGTTATTCAGAATGTGGTCGCAGCTCTTCAAGTTCTCCGAG
CTGTATATCAACTGAACGAACTGCAAAACGAGATCGGAAAGGCTCACGTGTGCAGACCAGAACAATAGTTA
TAAGCAAAAAATAACTTCGAGTATCCGAGTTTCCGTATCCGATACTTTCCAATCTTCACTTCAGTTTGTCC
TATGAATGTCGATTATGAAGAAGTATCATAACACTATGACTGTTATACAATAAAAGTTCGTAAATGTAAAA
AAAAAAAAAAAAAAGGG

Figure 3.16: Sequences obtained from one immunoreactive clones from the *A. vasorum* cDNA library encoding for a unique protein.

(pBluescript sequence and poly (A) tail removed)

Clone # 8- unique protein (606 bp)

```
1     ATGGATATCC CTGGCGTAGA TGGTGTACAT AATCGACAAA ACTGGTTTAA TCAGCAAAGA
61    TGGTGGCTAT TCATAAAACA GGCTGTATCG CTGCTTCTGT CCGAGAATAT GTTAAAAATA
121   CACCTCTTCA TCTACTGGAT CATTTGTCTG CTAACTACGA TGGAAGACCG ACAAAGTGTA
181   AATACGTTAG CTGTTATGGT TATCGCAATG TGTATTGTGT TTTGCATCGT CGCACTGGTG
241   ACAAGAAATG CCTGTTTTTT CTACCCGTTT CTTTTTCTGG TGACTTTTGA ATTTATGACG
301   TCGTTGGCCC TATTTTGCCT TATAGCGTTA AAAATTGGAT GGCCTCAAAA CTATGTGGCT
361   TTTGTAAAGG AAAATGAATT GGATTCTCTG CAGTATTTCG CGAAGTTCAC TCATGAAACA
421   AGTGAGGTGT CATGCTTGTG GGCTGGATTT CGAGCTTTGT ATTTTGCCCT AGTTGCTTTG
481   ACACTTGTAA AAAACATCAC CGATTACCAA GTCGACGCGC ATCATCGTCA ACACAATCGC
541   TACATTTATC CGCCGGTTCC TCCATATCCA CGTCTTGTCG CGATACCTGA AACCCGTAAT
601   GGGTAA
```

CHAPTER 4: GLUTATHIONE S-TRANSFERASE (GST) FUSION PROTEIN PRODUCTION-EXPRESSION OF RECOMBINANT PROTEIN, USING PROTEINS FROM THE IMMUNOREACTIVE CLONES OF *A. VASORUM* cDNA LIBRARY

4.1 ABSTRACT

This study continues the development of enhanced diagnostics for *Angiostrongylus vasorum* infection in dogs. The previous study identified 4 proteins reactive with rabbit antiserum generated against crude parasite somatic antigen. In the current study the most commonly occurring sequence, encoding for vitellogenin protein was chosen for expression and was inserted into a pGEX plasmid that was used to transform *E. coli* strain BL21. Protein expression as a glutathione S-transferase (GST) fusion protein was induced using IPTG and analysed by SDS-PAGE. The recombinant protein was identified by Western blot using rabbit *A. vasorum*-antisera. The fusion protein had a molecular mass of 77 kDa which represented the combined mass of the GST (26 kDa) and the partial vitellogenin protein (51 kDa). The fusion protein was purified using GST affinity resin. Several modifications to the protocol for eluting the protein of interest from an affinity column had to be made including adjustments to pH, salt concentration and Triton X-100 concentration. The generation of specific proteins should result in the development of antiserum that will be more sensitive than that obtained from crude *A. vasorum* antigen. Furthermore, this will potentially increase sensitivity of the sandwich ELISA and allow dependable production of reagents.

4.2 INTRODUCTION

Glutathione S-transferase (GST) fusion is a useful *E. coli* expression system for the production of large quantities of recombinant proteins. GST is a 26 kDa protein with a high affinity for the reduced form of glutathione. The GST protein is expressed in *E. coli* and purified using affinity chromatography with resin containing reduced glutathione (Saluta and Bell, 1998). The GST protein is then eluted from the resin in a nearly pure form. When GST is fused to another protein, this fusion protein can be likewise purified. The protein of interest is fused to GST, making subsequent specific cleavage of GST necessary to obtain a pure "native-like" protein of interest (Smith *et al.*, 1993). Proteins in the eluate of the resin are detected by SDS-PAGE and Western blotting using specific antisera. In this study, GST fusion proteins were produced by sub-cloning vitellogenin (the sequence of interest) into the pGEX-4T-1 plasmid containing a GST tag.

This study described a modified method for eluting and purifying a GST fusion protein from *A. vasorum*. A sequence encoding for a vitellogenin protein homologue was chosen from the *A. vasorum* cDNA library and expressed. This protein was chosen to be expressed as the fusion protein as it was the most common gene sequence found in our *A. vasorum* cDNA library. The GST vitellogenin fusion protein did not elute from the resin and modifications to the protocol were required for successful elution.

4.3 MATERIALS AND METHODS

Before protein expression was attempted, the chosen sequence encoding for vitellogenin protein was inserted into a different plasmid (pBlue-KX) and used to transform TOPO cells (*E. coli*, Invitrogen) to generate a sufficient amount of recombinant plasmid. The recombinant plasmid was purified by mini-prep and was sent for sequencing to verify that the chosen sequence had the same sequence as was described previously from the *A. vasorum* cDNA library in Chapter 3.

4.3.1 Vector and insert preparation and ligation

Clone # 4, with homology to vitellogenin protein, was chosen because it was the most common sequence in our cDNA library. A PCR reaction was performed (as was described in section 3.3.6.1) using the clone as a template with specific primers (Sigma) that were designed with forward primer: 5' CCAGGATCCATATCCAGCAAA CACTTGG 3' (the BamHI site is underlined) and the reverse primer: 5' TCTCGAGTAGCAGGCTATGGGTTGATTTTGC 3' (the XhoI site is underlined). Sterile water was used as a negative PCR control. The same PCR procedure was performed on both the clone and the amplified cDNA library using standard primers (T3 and T7). Following the PCR reaction, the PCR product was purified using the High Pure PCR product purification kit (Roche). To prepare the pBlue-KX plasmid for ligation, it was first double digested using 1/2 µl of each BamHI and XhoI restriction endonucleases in 2 x Tango buffer (Fermentas) in 30 µl for 3 h at 37°C. The reaction was terminated by heat inactivation at 80°C for 20 min. The same double digestion procedure was applied to the PCR product (insert DNA of interest). Following double

digestion, the plasmid and insert were purified separately using the High Pure PCR product purification kit (Roche) following the manufacturer's protocol. In brief, binding buffer supplied with the kit was added to the samples, transferred to the upper reservoir of the filter tube and centrifuged at 13,000 x g for 30 min in an Eppendorf 5415C centrifuge. Remaining impurities were removed by 2 washes with 500 µl washing buffer at 13,000 x g for 30 sec. The plasmid and the insert of interest were then collected by the addition of 50 µl supplied elution buffer and another 30 sec centrifugation at 13,000 x g. Purified insert and plasmid were ligated as follows: 4 µl of the insert was added to 3 µl of plasmid (pBlue-KX), 2 µl of 10 x ligation buffer (Fermentas), 1 µl T4 DNA ligase (Fermentas) and ddH_2O to a final volume of 20 µl. The ligation reaction was incubated at 22°C for 2 h, and heat inactivated at 65°C for 10 min.

4.3.1.1 DNA gel using SYBR Safe stain

One percent agarose gel electrophoresis with SYBR Safe stain (Invitrogen) was performed to verify the ligation reaction. One gram of agarose was added to 100 ml of 0.5 x TBS buffer into a flask, mixed gently and 3 µl of SYBR Safe stain (10,000 x concentrated in DMSO) was added. The mixture was heated in the microwave for 2 min or until the agarose was completely dissolved. The flask was cooled for 10 min and the gel was poured into a tray. Ten microliters of the ligation sample was loaded into the gel. The gel was run for 90 min at 120 V in 0.5 x TBS running buffer.

4.3.2 Transformation of competent cells and sequencing

The ligation product (recombinant plasmid-pBlue containing the insert) used to transform TOPO competent cells by adding 1.5 µl of ligation sample to the cells as follows: TOPO cells (Invitrogen) stored in -80°C were thawed on ice, and 1.5 µl of ligation sample was added. The reaction was incubated on ice for 30 min and then the bacterial cells were heat-pulsed by incubating the tubes for exactly 30 sec in a 42°C water bath. Two hundred and fifty microliters of room-temperature SOC medium (20 g Tryptone, 5 g Yeast extract, 0.5 g NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7) (Invitrogen) were added to the reaction tube and was incubated at 37°C with shaking at 200 rpm for 1 h. The sample was plated on LB agar plates (10 g Tryptone, 5 g Yeast extract, 10 g NaCl, 15 g agar, pH 7) containing Kanamycin (50 µg/ml) and the plate was incubated overnight at 37°C to allow colonies to form.

4.3.2.1 Miniprep plasmid purification and conformation of insert by restriction digest

Following growth, 12 isolated colonies were selected from the LB agar plate (from Section 4.3.2) and grown overnight at 37°C in 4 ml LB broth containing Kanamycin (50 µg/ml) with constant shaking set at 190 rpm. Miniprep was performed on all the selected colonies (Qiagen) with modification to the manufacturer's recommended protocol. Briefly, 1.5 ml of bacterial suspension was centrifuged at 12,000 x g in an Eppendorf 5415C centrifuge for 10 min at room-temperature in a 1.5 ml microcentrifuge tubes. The supernatant was discarded, and another 1.5 ml of bacterial suspension was added into the same tubes with the pellet and the tubes were centrifuged as before. The

pellet was resuspended in 250 µl of buffer P1 and 250 µl of buffer P2 was added and gently mixed by inverting the tubes 6 x. Then, 350 µl of N3 buffer was added and the tubes were immediately and gently inverted 6 x. The samples were centrifuged at 12,000 x g for 10 min at room-temperature and the supernatant from each tube was applied to the upper reservoir of the QIAprep spin column. The spin columns were centrifuged at 12,000 x g for 1 min at room-temperature and the supernatant was discarded. The spin columns were washed first by adding 500 µl buffer PB and centrifuged at 12,000 x g for 1 min at room-temperature and the supernatant was discarded, then the columns were washed again by adding 750 µl of buffer PE and centrifuged for 1 min at 12,000 x g. After the supernatant was discarded the spin columns were centrifuged for an additional minute to remove residual wash. The QIAprep columns were transferred to clean 1.5 ml microcentrifuge tubes. The plasmid DNA from all samples was eluted from columns with 50 µl of EB buffer incubated for 1 min at room-temperature and with centrifugation at 12,000 x g for 1 min at room-temperature. To confirm presence of the insert in the plasmid from each sample, all 12 samples were digested with BamHI and XhoI as above, and an agarose gel electrophoresis was performed as above.

4.3.2.2 Sequence analysis

Following the miniprep, 4 replicates of recombinant plasmids were chosen and sent for sequencing to the Guelph Molecular Supercenter Laboratory Services Division, University of Guelph. The sequences were analyzed for an open reading frame using BioEdit Sequence Alignment Editor. The Basic Local Alignment Search Tool (BLAST)

(Altschul *et al.*, 1990) was used to search for sequence similarities to vitellogenin and to confirm that all 4 samples had the same sequence as was described in Chapter 3.

4.3.3 Expression of vitellogenin protein

4.3.3.1 pGEX-4T-1 plasmid and insert preparation and ligation

For protein expression the pGEX-4T-1 plasmid was chosen because it allowed expression of the vitellogenin sequence in frame with the glutathione S-transferase generating a GST fusion protein. The recombinant pBlue-KX plasmid that had been verified for correct sequence were digested with BamHI and XhoI and electrophoresed. Following electrophoresis the DNA insert of interest was cut (from the gel) from each pBlue-KX plasmid. The 4 inserts were purified from the gel using High Pure PCR product purification kit. Double digestion of the 4 inserts was then performed by combining 6 µl of the purified sample, 6 µl of 2 x Tango buffer (Fermentas), 1/2 µl BamHI, 1/2 µl XhoI and ddH_2O for total volume of 30 µl. Double digestion of pGEX plasmid was performed by adding the following reagents: 1 µl of pGEX-4T-1 plasmid, 6 µl of 2 x Tango buffer (Fermentas), 1/2 µl BamHI, 1/2 µl XhoI and ddH_2O for total volume of 30 µl. These double digestion reactions of the inserts and the plasmid were then incubated for 3 h at 37°C and heat inactivated at 80°C for 20 min. Ligation reactions were performed on 4 chosen inserts as followes: 5 µl of insert, 7 µl pGEX plasmid, 5 µl ddH_2O then the ligation reactions were mixed gently and 2 µl of 10 x ligation buffer (Fermentas) and 1 µl T4 ligase (Fermentas) were added and the reactions were incubated for 2 h at 22°C, and heat inactivated at 65°C for 10 min.

4.3.3.2 Transformation of competent cells with pGEX

BL21 cells (*E. coli* strain, Novagene) while useful for protein expression are poorly transformed (Amersham and Novagene manuals). Before attempting expression, transformation of TOPO cells (*E. coli*, Invitrogen) was performed to generate sufficient quantity of recombinant plasmid. Ligation products (recombinant plasmids) were first mixed with TOPO competent cells (Invitrogen). TOPO competent cells were thawed on ice and 5 µl ligation reaction was added to the cells. The transformed reaction was incubated on ice for 30 min followed by heat-pulse treatment at 42°C for 30 sec then immediately transferred on ice. Afterwards, 250 µl SOC at room-temperature was added. The reaction was incubated at 37°C for 1 h with shaking at 200 rpm. Seventy microliters of the transformation reaction mixture was plated on LB agar plates containing 50 µg/ml ampicillin and these plates were incubated overnight at 37°C. Several colonies were chosen and a miniprep followed by agarose gel electrophoresis as described above was used to purify recombinant plasmids. Small amounts from the purified recombinant plasmids were diluted 1:100 and optical density (OD) values were obtained.

According to the manufacturer (Novagene), to transform BL21 (*E. coli* strain) competent cells only 1-10 ng of recombinant plasmid should be used. Following determination of optical density, 4 recombinant pGEX colonies with the highest OD reading were chosen. One sample was diluted at 1:5 with ddH_2O and the other 3 samples were diluted at 1:10 with ddH_2O. Transformation of BL21 cells (Novagene) was performed using the manufacturer's protocol with modifications as follows: BL21 competent cells were taken from -80°C storage and thawed on ice. One µl of each

diluted recombinant plasmid sample was added to 20 µl competent cells. The 4 reactions were incubated on ice for 30 min, and heat-pulsed for 30 sec in a 42°C water bath, and immediately placed on ice. Then 80 µl SOC (Invitrogen) medium was added to each reaction. The samples were incubated at 37°C for 1 h with shaking at 200 rpm. Eighty microliters from each of the transformation reactions were plated on 4 Luria Bertani (LB) agar plates containing 50 µg/ml ampicillin. The plates were incubated overnight at 37°C. Two colonies from each plate were chosen, and miniprep, double digestion and agarose gel electrophoresis were performed as described above to verify the subcloned DNA inserts.

4.3.3.3 Expression of proteins using isopropyl-β-D-thiogalactopyranoside (IPTG)

The production of fusion proteins was performed as described in Frangioni and Neel (1993): Two colonies from each of the 4 agar plates that were prepared previously with BL21 were grown overnight. Each colony was mixed with 2 ml LB broth (10 g NaCl, 10 g Tryptone, 5 g Yeast extract, pH 7) containing ampicillin (100 µg/ml) in 10 ml glass tubes and incubated overnight at 37°C with shaking at 185 rpm. Two milliliters of the bacterial growth were transferred into flasks containing 100 ml LB broth with ampicillin (100 µg/ml) and incubated at 37°C with agitation set at 185 rpm for 90 min. Following incubation, expression of the fusion gene was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) that was added to a 0.1 mM final concentration. The flasks were incubated for 4 h at 37°C with shaking at 185 rpm. The bacterial suspension was then transferred into 50 ml plastic bottles and the cells were harvested by centrifugation for 10 min at 7000 x g. The supernatant was discarded and the pellet was washed with 6

ml ice cold STE (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8) buffer by centrifugation at 7000 x g for 5 min. The supernatant was discarded and 6 ml of ice cold STE buffer containing 100 µg/ml lysozyme was added to the bacterial pellet which was then resuspended. This pellet was then transferred into
15 ml polystyrene tubes and incubated on ice for 15 min. Following the incubation 5 mM of DTT (diothiothreitol) was then added, the suspension was gently mixed, and the bacteria were lysed by the addition of sarkosyl (N-laurylsarcosine) to a 1.5% final concentration (10% stock solution in STE buffer), with vortexing for 10 sec. The cells were then sonicated (Fisher Sonic Dismembrator model 100) on ice using short bursts of 10 sec allowing the protein (bacterial suspension) to cool on ice between each burst. Each sample was sonicated for 1 min (power level 4, 50% duty cycle). After sonication, each lysate was clarified by centrifugation for 5 min at 10,000 x g at 4°C. The supernatant was transferred into clean 15 ml polystyrene tubes and Triton X-100 was added to give a final concentration of 4% (Triton X-100 was in stock solution of 10% with STE buffer). The tubes were then mixed and vortexed for 10 sec and incubated on ice for 15 sec. Protein expression was confirmed by 12% SDS-PAGE gel stained with Coomassie Blue dye.

4.3.3.4 SDS-PAGE gel and Western blot analysis

4.3.3.4.1 SDS-PAGE gel

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Laemmli, 1970) using a 12% running gel, and a 5% stacking gel with the Mini-Protean II system (Bio-Rad). Two gels were prepared and all 8

samples from the last procedure were incubated on ice. Twenty microliters from each sample was diluted 1:4 with 2 x SDS-PAGE sample buffer and boiled for 10 min. After cooling, 10 µl from each sample was loaded into wells from each side of the two gels and one well from each gel was loaded with 5 µl of molecular weight marker. The gels were placed into Bio-Rad Mini-Protean II electrophoresis chamber and run for 1 h at 100 V with 5 x running buffer. The gels were then removed from the chamber and one gel was stained with Coomassie Blue to verify the presence of protein in the samples. Gels were stained for 45 min with gentle agitation and destained with water for 40 min with agitation. The gel was then washed with water to stop the reaction and remove residuals. The second gel was retained for Western blotting.

4.3.3.4.2 Western Blot

4.3.3.4.2.1 Excretory/Secretory rabbit polyclonal antisera production

Western blotting was performed using rabbit polyclonal antiserum produced against excretory/secretory (ES) antigens of *A. vasorum*. To prepare this antiserum, the ES antigen was generated as follows: adults *Angiostrongylus vasorum* were collected from experimentally infected dogs and rinsed 2 x with saline containing 5% penicillin/streptomycin (pen/strep) (Sigma). The parasites were placed in a fresh Petri dish with L15 (Leibovitz's) medium (Invitrogen) and 1% pen/strep and incubated for 48 h at 37°C. The supernatant media was collected and centrifuged at 1500 x g for 10 sec. This supernatant containing the ES antigen was collected and filtered using a 0.2 µm syringe filter and the ES antigen was collected into a new tube. Female New-Zealand White rabbits were immunized with the ES antigen. The antigen was mixed 1:1 with

adjuvant (MPL/TDM/CWS, Sigma) to give an inoculum of 250 μg of protein in 0.6 ml. This was administered subcutaneously and evenly distributed to the hind legs and the neck. Three immunizations were given over a 3 month period. Two weeks after the third immunization, the rabbits were euthanized and a final bleed was performed. Serum was collected and stored at -20°C. This ES polyclonal serum was used in the Western blot to identify the GST fusion protein. A crude rabbit polyclonal antiserum that was prepared earlier (Section 2.3.3) and used in the sandwich ELISA test was also used in the Western blot to identify the GST fusion protein of interest.

4.3.3.4.2.2 Western Blot using an excretory/secretory rabbit polyclonal antiserum and a crude rabbit polyclonal antiserum

The SDS-PAGE that was not stained was used in a Western blot. The gel was equilibrated with transfer buffer (25 mM Tris-HCl, 192 mM glycine, pH 8) and a nitrocellulose membrane was placed onto the gel. Location of wells was marked on the filter by cutting the upper right hand corner. The sandwich of gel and membrane was mounted into the Mini-Protean II electrophoresis apparatus. An ice pack was added to maintain temperature during transfer. The proteins were transferred from gel to membrane by applying 120 V for 90 min. After transfer, the remaining binding capacity of the nitrocellulose membrane was blocked by incubating the membrane overnight at 4°C in 2% BSA in TBS buffer (0.5 M NaCl, 20 mM Tris-HCl, pH 7.5). The membrane was washed with TTBS (TBS with 0.05% Tween 20) for 5 min for a total of 3 washes. The membrane was then incubated with ES rabbit polyclonal antiserum diluted at 1:1000 with 1% BSA in TTBS for 1 h with gentle agitation at room-temperature. The

membrane was washed with TTBS for 5 min for a total of 3 washes. Following the washes, the membrane was incubated for 1 h with agitation at room-temperature with rabbit anti-mouse IgG labeled with horseradish peroxidase (HRPO) diluted 1:1000 with1% BSA in TTBS. Following incubation the membrane was washed 3 x with TTBS followed by 2 washes with TBS for 5 min each. The 4-chloro-1-naphthol-H_2O_2 substrate buffer was then added. The colour reaction was left to develop with gentle agitation for 7 -10 min or until the desired level of signal was achieved after which the reaction was stopped by rinsing the membrane in water. The same procedure was performed using the crude rabbit polyclonal antiserum that was used before in the sandwich ELISA test.

4.3.4 GST Protein Fusion

4.3.4.1 Batch purification of GST fusion proteins

Glutathione S-transferase (GST) fusion proteins can be purified directly from cell lysates using glutathione affinity chromatography resin, and this approach was used on the proteins that were expressed using IPTG (Section 4.3.3.3). Cleavage of the desired protein from GST was achieved using a site specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX-4T-1 plasmid. Glutathione Sepharose 4B kit (Amersham) was used to purify the protein of interest. The procedures were modified from the manufacturer's protocol as follows.

4.3.4.2 Preparation of GST slurry

To prepare the slurry for use, 1.33 ml of the 75% sepharose slurry (Amersham) was transferred to a 15 ml polystyrene tube and centrifuged at 500 x g for 5 min. The supernatant was discarded and 10 ml of cold 1 x PBS was added to the pellet. The tube was mixed by inverting it gently 3 x, and then centrifuged at 500 x g for 5 min. The supernatant was discarded and 1 ml of cold 1 x PBS was added and the 50% slurry was ready to use.

4.3.4.3 Batch purification of GST fusion proteins

Cell lysates from the IPTG induced cultures (Section 4.3.3.3) in 15 ml polystyrene tubes were used for purification. Eight hundred microliters of the 50% slurry was added to each tube and the cell lysate samples were incubated with gentle agitation at room-temperature for 30 min. Following incubation, the samples were washed with 4 ml (10 bed volumes) of 1 x PBS by centrifugation at 500 x g for 5 min and this wash was repeated twice.

4.3.4.4 Elution of GST fusion proteins from glutathione beads

Problems were encountered in eluting the fusion proteins. According to the manufacturer's protocol, an elution buffer of 10 mM reduced glutathione in 50 mM Tris-HCl and pH 8.0 should be effective in eluting proteins (Amersham). However, in our experiment the elution buffer did not release the proteins from the affinity resin. The addition of salt (1 M NaCl and 2 M NaCl) and increasing the pH to 9 did not elute the proteins. The manufacturer's suggestion (Amersham) to decrease salt concentration and

to increase the reduced glutathione to 25 mM still failed to produce elution. The suggestion from Frangioni and Neil (1993) for use of 0.1% TritonX-100 did not improve elution. Therefore we devised the following recipe for elution buffer: 160 mM NaCl (Fisher), 80 mM Tris (Fisher), 0.2% TritonX-100 (Fisher) and 40 mM reduced glutathione (Sigma) and with pH 9.5. One ml of this elution buffer was added to each sample (from Section 4.3.4.3) and the tubes were mixed gently and centrifuged at 500 x g for 5 min at room-temperature. The supernatant was transferred to a new 4 ml polystyrene tube. The elution procedure was repeated twice with a total of 3 eluates. Twenty microliters of the eluates were mixed with sample buffer and run on SDS-PAGE gel. The rest of the sample was stored in -80°C.

4.3.4.5 Thrombin cleavage

A sample of one bacterial lysate was mixed with the glutathione slurry (Section 4.3.4.3) and was used for thrombin cleavage to generate the protein of interest free of GST fusion protein. Five hundred units of thrombin 500 (Amersham) was first dissolved in 0.5 ml of cold 1 x PBS as a stock solution. From the stock solution 50 µl was mixed with 950 µl cold 1 x PBS and used as working solution. Eight hundred microliters of thrombin solution were taken from the working solution and added to the bacterial lysate sample that was mixed with the 50% GST slurry, and incubated for 10 h. Following incubation, the tube was mixed gently and centrifuged at 500 x g for 5 min at room-temperature. The supernatant was removed and checked on a SDS-PAGE for the presence of GST, and the pellet was kept. The elution buffer that was used above was

added to the pellet. Following the same procedure of elution, the final eluates were evaluated and analyzed using SDS-PAGE with Coomassie Blue staining.

4.3.4.6 Elution of GST fusion proteins using standard sepharose beads

One IPTG induced cell lysate sample (Section 4.3.3.3) was mixed with standard sepharose beads that did not contain reduced glutathione (Amersham). One ml of sepharose beads was added to the cell lysate and incubated with gentle agitation at room-temperature for 30 min. Following incubation the sample was washed with 4 ml (10 bed volumes) 1 x PBS by centrifugation at 500 x g for 5 min at room-temperature. This wash was repeated twice. The sample was eluted using the modified elution buffer following the same elution procedure as above. Both eluted protein and the beads themselves were placed in SDS-PAGE sample buffer and subjected to SDS-PAGE.

4.3.5 Concentration of GST fusion proteins

Fusion proteins were concentrated 2 x using Centriplus concentrators (Amicon) following the manufacturer's protocol.

4.3.6 Protein Assay of the GST fusion Proteins

The protein content of eluted fusion proteins was determined using a Bradford assay (Bio-Rad) procedure for microtiter plates following the manufacturer's protocol.

4.4 RESULTS

4.4.1 Immunoreactive clone using specific primers

The sequence from the most common immunoreactive clone (# 4 from Chapter 3) with homology to vitellogenin protein from *A. vasorum* cDNA library was chosen. A PCR reaction using the specific primers generated a product with ~ 1600 bp (Figure 4.2). This PCR product was purified and ligated into a pBlue-KX plasmid. The ligated product (plasmid and insert) was sent for sequencing and was the same sequence from Chapter 3, i.e. vitellogenin protein with 76% similarity to *C. elegans* vitellogenin protein (Figure 4.1). After verifying the correct sequence and performing another restriction enzyme digestion and gel electrophoresis, the insert was cut from the gel (from pBlue-KX plasmid) and pGEX-4T-1 (Figure 4.3) was used for cloning the sequence of interest (DNA insert). The insert was successfully ligated into the pGEX-4T-1 and the recombinant pGEX-4T-1 plasmid was verified by restriction digest with BamHI and XhoI. The replicates of the recombinant plasmid can be seen in Figure 4.4, lanes 4-7, where bands representing the recombinant plasmid and the uncut pGEX-4T-1 can be seen as a larger band (4900 bp) with no insert in lane 8.

4.4.2 Expression of proteins using IPTG

The recombinant plasmid selected was expressed by inducing the taq promoter of pGEX-4T-1 with IPTG. The BL21 bacterial cells transformed with the plasmid alone and induced with IPTG produced a GST protein of 26 kDa (Figure 4.5). The protein of interest fused with GST showed a molecular mass of 77 kDa as was expected from the

combination of the protein of interest which was calculated to be 51 kDa and the GST which is 26 kDa (Figure 4.5).

4.4.3 Elution of the fusion proteins from the glutathione beads

Using the modified elution buffer that was developed in this study, the fusion proteins were eluted from the glutathione beads. The GST negative control band in lane 4 is very thick and clear at 26 kDa and lane 5 shows the GST vitellogenin fusion protein with a 77 kDa band which is clean with no contamination (Figure 4.5).

4.4.4 Western blot

A Western blot was performed with the same crude polyclonal rabbit antiserum that was used in the sandwich ELISA, and in screening the cDNA library and also with the antiserum directed against the ES antigen. In Figure 4.6, Western blotting using the crude rabbit polyclonal antiserum generated bands corresponding to the GST vitellogenin fusion protein. In the same figure, reactivity with the GST is not evident although some bands representing contaminating bacterial proteins are seen. After purification on the affinity column, these non-specific reactions do not appear. To remove non-specific reactions this antiserum would need to be absorbed with bacteria. Rather than using absorption, after initial attempts with the crude polyclonal antiserum we used ES polyclonal rabbit antiserum which showed much reduced cross-reactivity with *E. coli* and fewer bacterial bands were observed. This is evident in Figure 4.7 in which the negative control cell lysate has very few bacterial bands, as well as the GST vitellogenin fusion protein. Western blot showed that the GST vitellogenin fusion

protein could be specifically detected with the rabbit anti-ES polyclonal antibody. The rabbit anti-ES polyclonal antibody did not react with the GST protein. A single band was detected at 77 kDa in lane 5, which is the appropriate GST vitellogenin fusion protein mass (Figure 4.7). The band on the blot was prominent and with no smears, indicating proteins were not contaminated with bacterial antigens (Figure 4.7).

4.4.5 Thrombin cleavage

The GST vitellogenin fusion protein was cleaved by thrombin in order to eliminate the GST and elute the protein of interest. This generated proteins of 51 kDa and 26 kDa corresponding to the partial vitellogenin protein and GST respectively. However, due to affinity of the protein to the beads, much of the cleaved protein remained adherent to the glutathione beads (Figure 4.8).

4.4.6 Elution of GST fusion proteins using standard sepharose beads

When bacterial lysates were mixed with sepharose beads without glutathione residues, it was clear that the GST fusion protein was adhering to the beads. Even after elution with the modified buffer, GST fusion protein remained attached to the beads and this was visualized by subjecting beads to SDS-PAGE (Figure 4.9).

4.4.7 Protein assay and concentration of fusion proteins

The Bradford protein assay indicated 400 μg per ml. Following the 2 x concentration, GST fusion proteins were 1059 μg per ml.

4.5 DISCUSSION

Glutathione S-transferase (GST) was originally cloned from the parasite *Schistosoma japonicum* and has a range of applications since its introduction for synthesis of recombinant proteins in bacteria (Smith *et al.*, 1993). Some general applications of GST include; generation of reagents, antigen production which can be used to generate antibodies, production of antigens for in-vitro diagnosis, and generic tools for protein expression and purification. Protein purification is important when the protein of interest is required for production of specific antibodies. The protein of interest is produced as a fusion with GST, making subsequent proteolysis necessary to obtain a pure "native-like" protein of interest (Smith and Johnson, 1988). The coding sequence for the GST fusion protein is cloned into an isopropyl-β-D-thiogalactoside (IPTG)-inducible expression vector. This fusion protein is expressed in bacteria and purified by affinity chromatography on glutathione-agarose beads (Saluta and Bell, 1998). In general, the sequence encoding the GST protein is incorporated into an expression vector, generally upstream from the multi-cloning site. The sequence encoding the protein of interest is then sub-cloned into this vector. Induction of the vector results in expression of the protein of interest fused to the GST protein. The GST vitellogenin fusion protein produced can be collected and purified. Purification of the GST vitellogenin fusion protein is facilitated by the affinity of the GST protein for glutathione residues. Glutathione residues are coupled to a resin and the expressed protein product is brought into contact with the resin. The GST vitellogenin fusion protein will bind to the glutathione-resin complex, and all other non-specific proteins can be washed off. The GST vitellogenin fusion protein can then be released from the

resin using an elution buffer (Majka *et al.*, 1997). The GST can be removed from the protein of interest by using different enzymes such as thrombin, which cleave specific sites between the GST and the protein of interest (Dian *et al.*, 2002).

The target proteins in this study are cell lysates. When the cell lysate-GST fusion proteins were incubated with glutathione-agarose beads they adhered as expected. However, several problems were encountered with the protein elution protocol suggested by the manufacturer, by Saluta and Bell (1998), by Dian *et al.* (2002) and by Guan and Dixon (1991), in that the suggested elution buffer was not able to release the GST fusion protein from the affinity resin. The apparent affinity of the protein to the glutathione beads was tested with sepharose only since the matrix for the reduced glutathione affinity resin is largely sepharose. The GST vitellogenin fusion protein did adhere to the sepharose beads, suggesting that the glutathione beads containing sepharose will bind non-specifically to the GST vitellogenin fusion protein. This needs to be considered in elution strategies.

During this study, a method which utilizes the sodium salt of the alkyl anionic detergent N-laurylsarcosine previously demonstrated to effectively solubilize GST fusion proteins while maintaining protein activity (Frangioni and Neel, 1993). Furthermore, the use of Triton X-100 can facilitate elution of proteins from glutathione beads (Frangioni and Neel, 1993). The elution step was attempted several times utilizing various salt concentrations, pH level increases, adding Triton X-100, and temperature variations. In this study, we had to increase salt concentrations, increase the pH, increase concentration of Triton X-100, increase the molarities of the reduced glutathione buffer in the elution buffer and incubate the GST vitellogenin fusion proteins

at room- temperature in order for the GST vitellogenin fusion proteins to be eluted from the glutathione beads. Using this modified elution buffer the GST vitellogenin fusion proteins were eluted from the resin probably by preventing the non-specific binding of the GST vitellogenin fusion protein to the sepharose resin, however some proteins still remained adherent to the beads. This was evident after elution when the washed beads were subjected to SDS-PAGE and still generated bands with characteristics of the GST vitellogenin fusion protein. Nonetheless, we did elute the fusion protein from the affinity resin. We found the GST vitellogenin fusion protein to be ~ 77 kDa and consisting of the protein of interest, which is 51 kDa, and the GST, which is 26 kDa. The SDS-PAGE and the Western blot both showed a clean band with an apparent molecular mass of 77 kDa.

To retrieve the protein of interest, it must be separated from the GST. The pGEX-4T1 produces a GST fusion protein that contains a thrombin-cleavage site between the GST and the target protein. This is intended to separate the GST and the target protein. The vitellogenin protein was cleaved and eluted. However, a considerable amount remained adherent to the beads. This protein may be compact and difficult to elute or, since it is a yolk protein, it may be unusually inclined to stick to the sepharose that glutathione beads contain. Problems that occurred with elution using the manufacturer's GST kit at the time prevented the use of the purified protein in the sandwich ELISA test which was previously developed. The GST vitellogenin fusion protein however can be used in the sandwich ELISA (protein of interest and the GST) and a GST control could be run in the test. The cleavage conditions appear to depend on the protein of interest and should be optimized for individual proteins with consideration

toward protein instability, cleavage efficiency, aggregation and purity of the final cleaved protein product.

Others have encountered problems with eluting GST fusion proteins from the affinity beads. Abath *et al.* (1997) conducted a study on expression of recombinant antigens in *E. coli*. They attempted to develop antibodies to GST fusion proteins of *Schistosoma mansoni*. In their study they had problems with thrombin cleavage because the GST fusion protein that they developed was not completely eluted from the beads and was partially cleaved with thrombin within the agarose affinity matrix (Abath *et al.*, 1997). The antibody was developed successfully against the GST fusion protein and control anti-GST was tested on Western blot (Abath *et al.*, 1997). Mice that were successfully immunized with *Entamoeba histolytica* GST fusion proteins produced specific antibodies for the target protein (Lopez-Monteon *et al.*, 2003).

Schnieder's study (1992) used GST fusion proteins from *Dictyocaulus viviparus* and the fusion proteins were applied on an ELISA test as well as purified protein (with no GST) and both recombinant antigen preparations showed high specificity for antibodies to *D. viviparous*. The study by Schnieder (1992) showed that GST fusion proteins are specific so that, although we could not purify the proteins from the GST in the present study, the fusion proteins can be considered to be a purified and specific protein to *A. vasorum*. Thus, using the SDS-PAGE and Western blot, the rabbit polyclonal antisera did not react with the GST but reacted against the GST vitellogenin fusion protein only. A mouse or a rabbit immunized with the protein could be used for production of monoclonal or polyclonal antibodies respectively which might be effective in the sandwich ELISA.

In conclusion, the use of specific antibodies could increase the sensitivity of the ELISA test used to evaluate *A. vasorum* infection in dogs. Furthermore, production of such antibodies could provide a more stable and reliable supply of reagents for diagnostic tests, which would allow the sandwich ELISA test to be used in routine diagnosis of *A. vasorum* infected dogs.

4.6 REFERENCES

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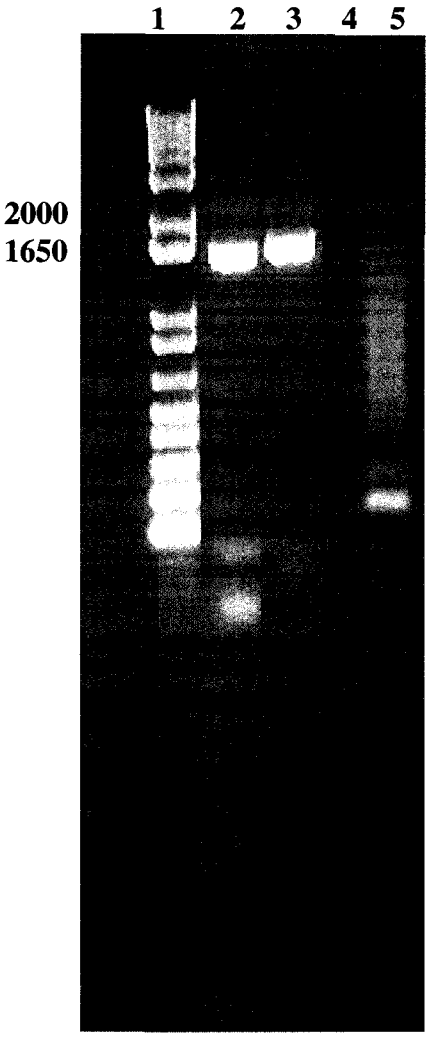
Figure 4.1: Sequence of immunoreactive clone # 4 (1618 bp)-coding for partial vitellogenin protein, using specific primers to clone into plasmids (pBlue-KX and pGEX).

```
1     ATCAGTGACA TGAAACCAGG ATCCATATCC AGCAAACACT TGGAACTTTT AGAGATGCCT
61    GTTCAATTTG TCTACAAAAA TGGAATGGTC GCTGAGCTGC AGTTCTCGGA AAAAGAAGAG
121   GCATGGTCAG CGAACATAAA GCGCTCCGTT ATCAACATGC TTCAAATTAA TCTTCACAAA
181   GTCGGAAAAA TCGATGAGAC TAACGTAGAT GGAGCATTGA TGGTTCGCGA AAACGACTTC
241   TTCAATGCTA ACGAGAGAAC TATCGAAGGG GACTGCGAAG TGGCCTACAC AGTTCTTAAG
301   AAGAAAGATG ACATCACTGA GGTTACCAAG TCAGTAAACT TCAATAAATG TTCTCGCCGT
361   CCACAGGCCA AATACAATTT CCGCTATTTA TCTGAATGTC GCGACTGCAA TGATGCCGAC
421   AACTTTGAGC CGAATACTGT CTATAACTAT GTACTAGAGA ACAACGGCTT GAAGAAAGTT
481   GAGGTCATGA GTGTGTACAC CGTTACCATT GACAACCAAC CTGTAATGAA GACCGAAGTC
541   CGCGCTAGAC TCAGCCTCGA AGACGTCAGA AAGATCAGAC AGCAGTTCGA GTCATCCAGA
601   GGCAAGACCG AAGGCCTAAT TTATTCCAAT GAGATGGAGA AACAGATTGA ACGTTTCTAT
661   ATGTATGGAG ACGACACAGA GGTTCTTCCA TATGAGCGTG TTACCGACAA AATTGAAGCC
721   ATTCATAAGA TCATTGATGG GATCAGAGAG CCAAAGGAGA ACAACTACGA AAACACTGTC
781   CTCGTGTCCC GTCTTGTTTC CATACTTCGT ATGTGCTCTC TCCGCGAACT GAGTGCTGTC
841   CACTCCGACA TCTATATGAA GGGTGATGAG CGCCTAAGGG CTATAATGGA GTATTCGTTG
901   GCCATCGCTG GTACGAAAAA CACTGTTACT CACCTACTTC GCCACGTGCA GAAGGAAGAT
961   GTCAAGACCT CCAGAATTGT GGGCCTTCTC AAGTCCATCC AAGAGCTGCC GTATCCTTCT
1021  TCCAAAGTCG TTGAAGAACT ACTCCGCTTC GCTGATAGCG GCGTTGTAAA ACGTTCTCCA
1081  GCTGTCCATC AGACCACATG GCTTGCAATA GGATCAGTCA TGCGCGGAGT AGTTGGAGAC
1141  ACCATGGATG AGAACCTTTT GGTCGAGAAT CACCGCGGAC TGAAACAGAA ATACCTCAAC
1201  ATGTTATTGA AGCAGTTTGA AAAAGCCGAC AAAATTTACG AAAAGGTGCT CGCTCTCAAG
1261  TCCCTCGCAA ACGCCGGAAT AGACCTATCT GTCCATGAAC TCGAAAAGAT TATCATGAAC
1321  AAGCATGAAG AGTTGCCTGT TCGTATGGAA GCGATCGACG CTCTCCGCCT GCTCAAGGAT
1381  TCCATGCCTC GAAAACTGAA ATCTGTTCTG ATGCCTGTTT ACAAAAACCG TCTCGAACAT
1441  CCTGAACTTA GAATGGCAGC TCTTGTCCGC ATTATGCACA CCCTACCTCA ACAATCTGTC
1501  GTCGCTCAGA TAGTCTCCAC CATGGAGCGT GATTCTAATC AGCAGGTAGC TGCCTTTACC
1561  TATGACTTGC TAAACTCGTT TGCAAAATCA ACCCATAGCC TGCTACTCGA GACTCGCC
```

Note: Bold nucleotide sequence showing the specific primers, underlined sequence showing the BamHI site and XhoI site

Figure 4.2: An Agarose gel showing amplicons derived from immunoreactive clone # 4 from the *A. vasorum* cDNA library, using specific primers and standard T3 and T7 primers. Also showing the amplified *A. vasorum* cDNA library using T3 and T7 primers.



Note: **Lane 1**- 1 kb plus DNA ladder, **Lane 2**- Arrow indicates a PCR product from the selected immunoreactive clone # 4 from *A. vasorum* cDNA library using specific primers, **Lane 3**- PCR product from the selected clone (# 4) using the standard T3 and T7 primers, **Lane 4**- negative PCR control, using water as a template, **Lane 5**- PCR product from *A. vasorum* cDNA library using standard T3 and T7 primers.

Figure 4.3: Vector map of pGEX-4T-1, showing restriction sites, thrombin-cleavage site, glutathione S-transferase tag, multiple cloning site (MCS) and control elements regulating the expression of the vector.

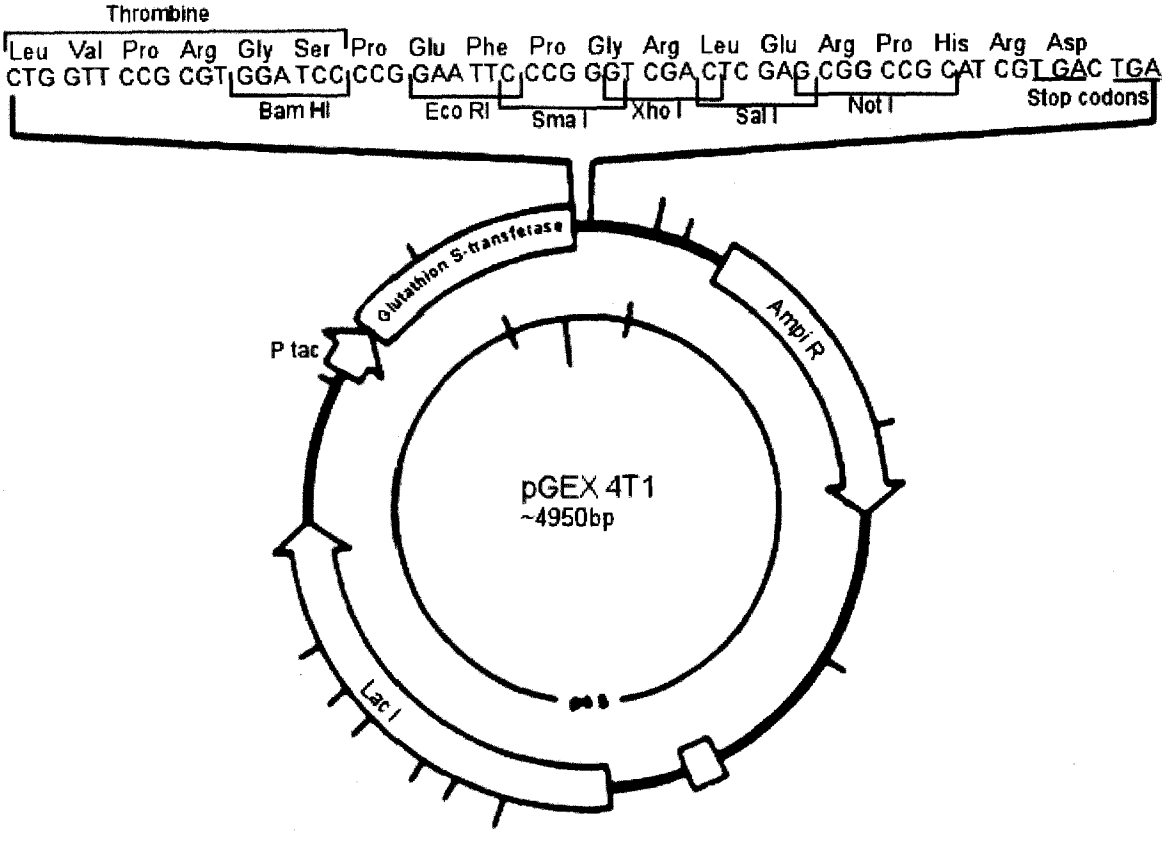
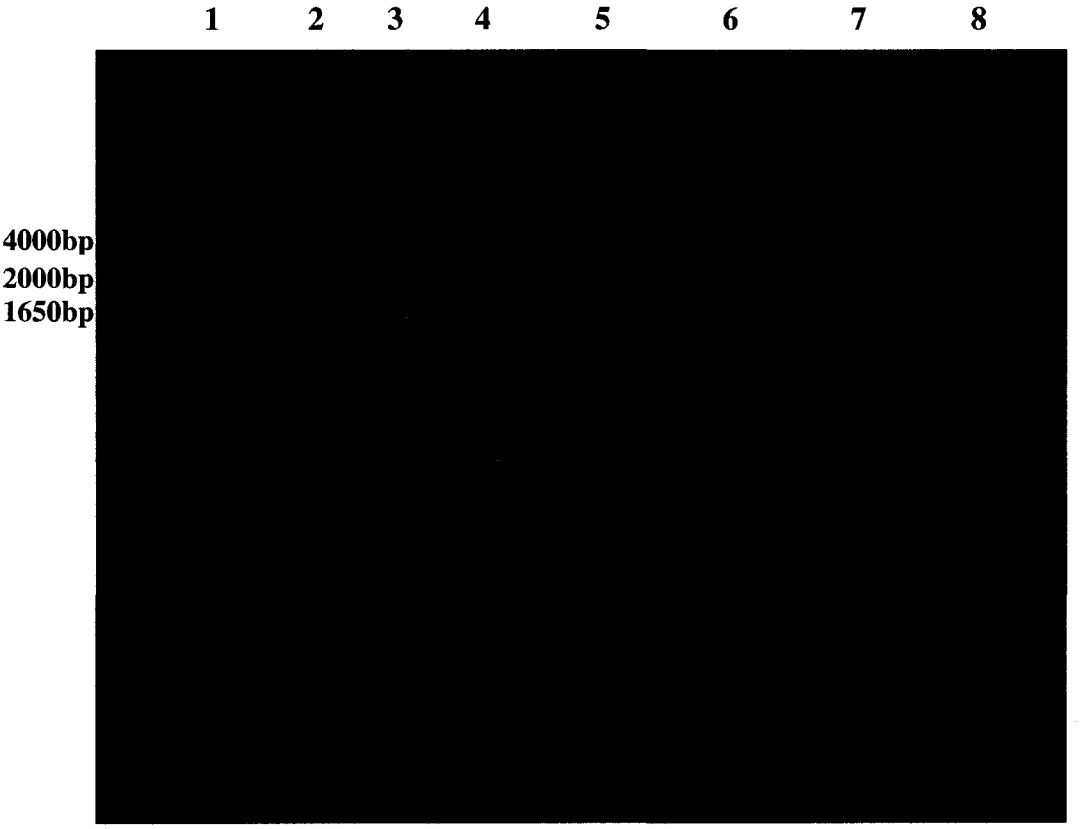
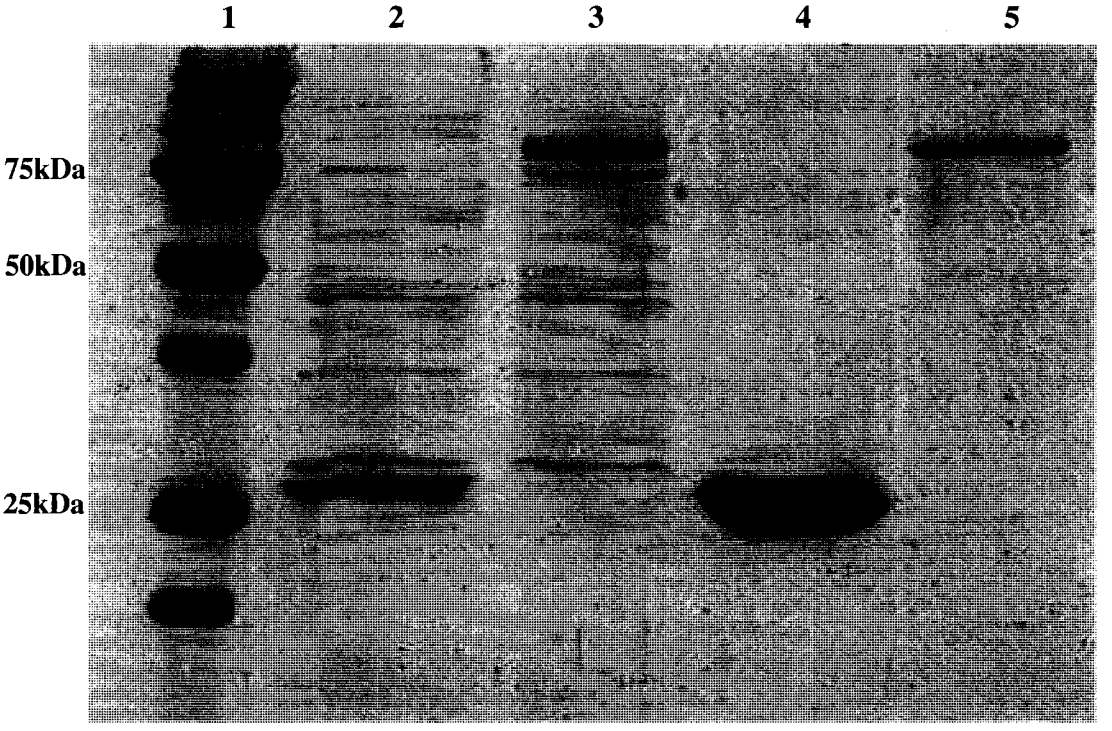


Figure 4.4: Agarose gel of restriction digests of recombinant pGEX-4T-1 using BamHI and XhoI.



Note: **Lane 1**- 1 kb plus DNA ladder, **Lanes 2 and 3**- empty, **Lanes 4, 5, 6 and 7**- replicates of immunoreactive clone # 4 (protein of interest) restriction digested from pGEX-4T-1 using BamHI and XhoI, **Lane 8**- pGEX-4T-1 with no insert, showing band with greater than 4900 base pairs.

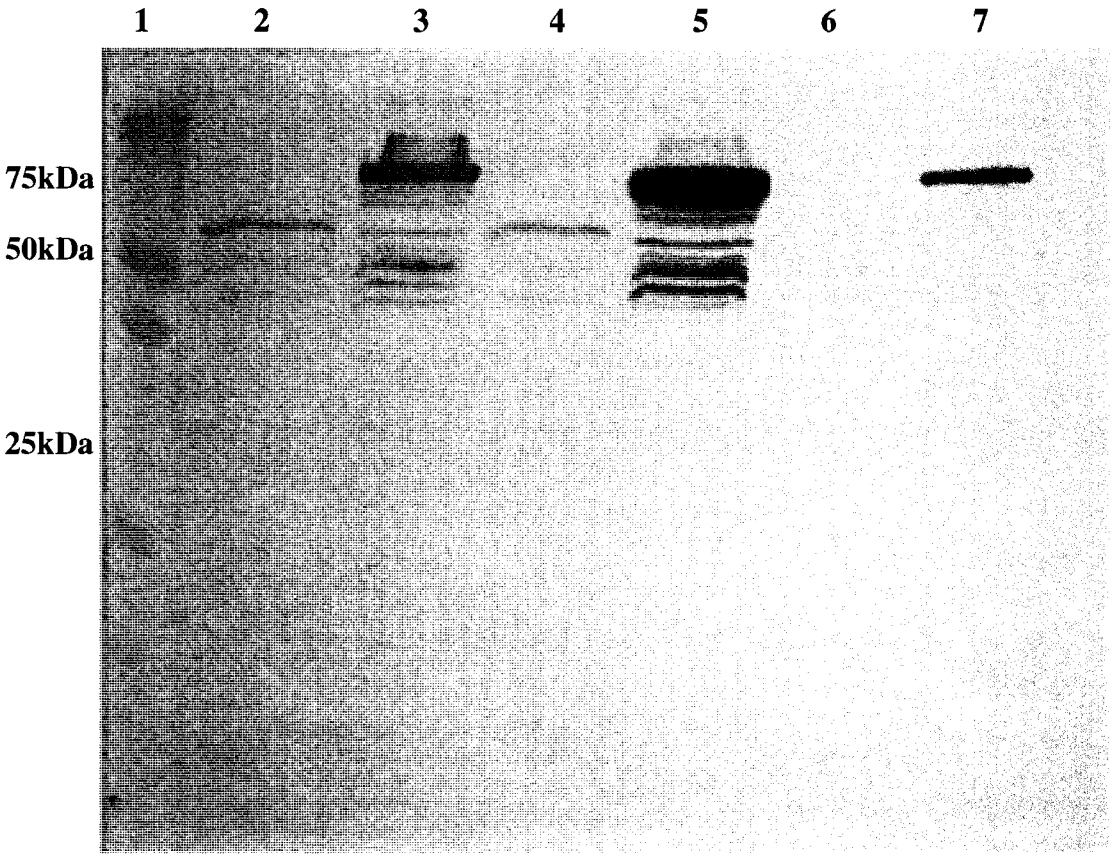
Figure 4.5: SDS-PAGE of cell lysates from pGEX-4T-1 transformed BL21 cells and proteins derived from glutathione affinity purified GST vitellogenin fusion proteins.



Note: **Lane 1**- Marker, **Lane 2**- negative control (plasmid with no insert)- IPTG Induced control GST protein cell lysate; showing bacterial bands and thick band of GST at 26 kDa, **Lane 3**- IPTG Induced GST vitellogenin fusion protein cell lysate, showing a thick band at 77 kDa and bacterial bands. **Lane 4**- Control GST protein elution after purification with glutathione beads. **Lane 5**- GST vitellogenin fusion protein after elution and purification with glutathione beads.

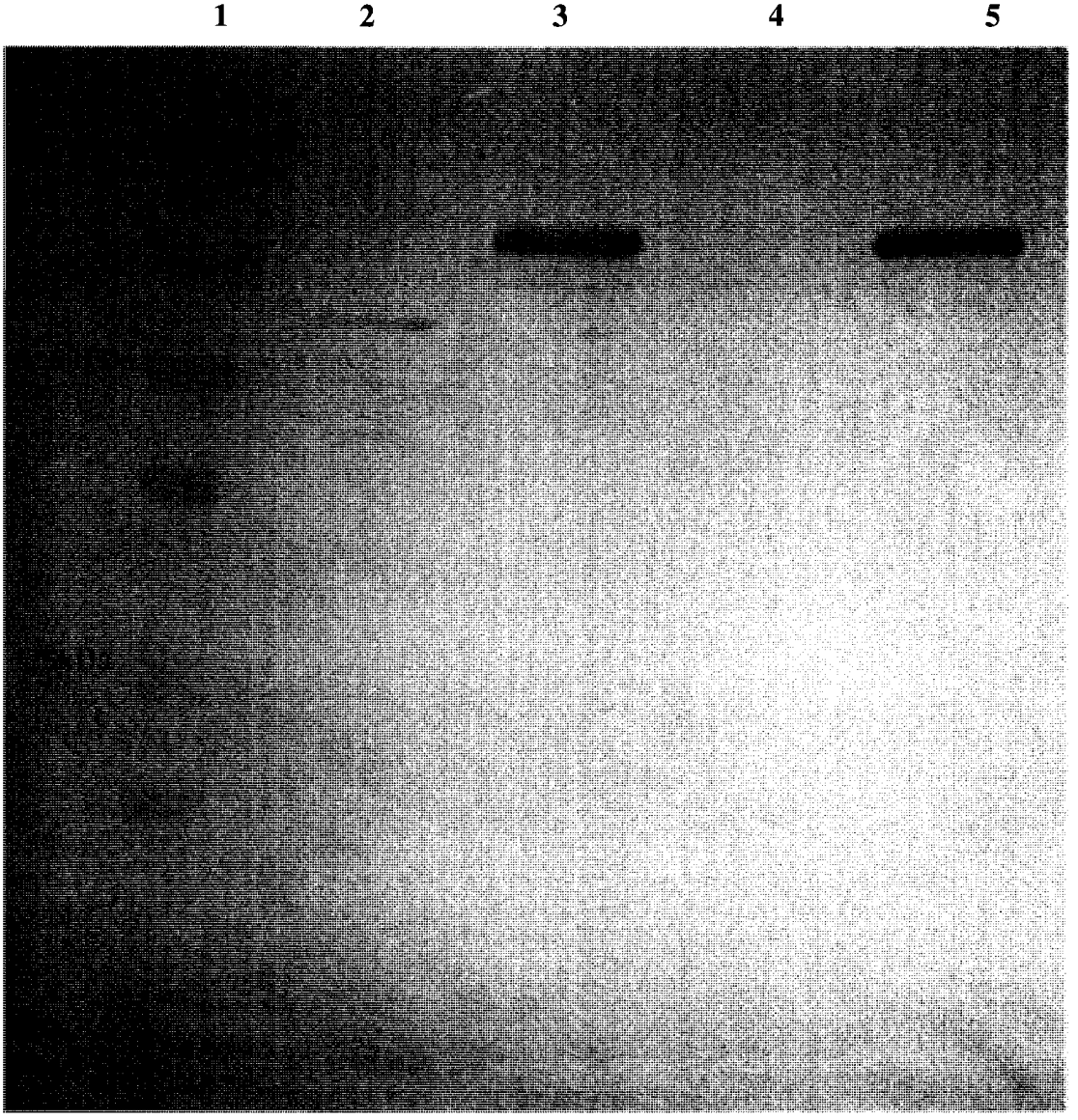
- The clean bands indicate eluted proteins are purified and clean from bacterial contamination

Figure 4.6: Western blot of GST vitellogenin fusion proteins using crude polyclonal rabbit antiserum.



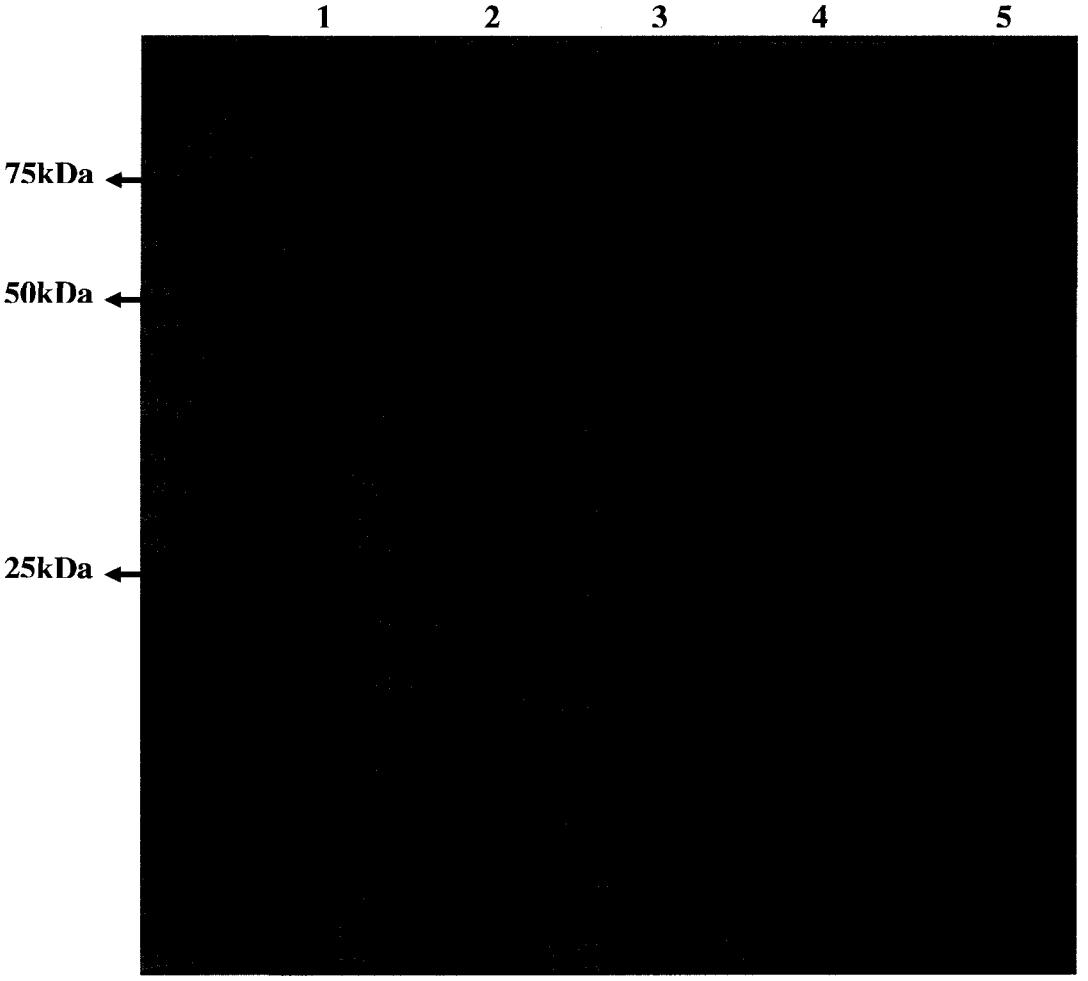
Note: **Lane 1**- Marker, **Lane 2 and 4**- negative control (IPTG Induced control GST protein cell lysate) and some bacterial bands are seen, **Lane 3 and 5**- GST vitellogenin fusion protein (IPTG Induced GST fusion protein cell lysate) and some bacterial bands, **Lane 6**- negative control after elution, **Lane 7**- GST vitellogenin fusion protein after elution, there are no bacterial bands following the elution.
The absence of bands in lane 6 indicates that the crude rabbit polyclonal antiserum did not react with the GST, demonstrating that it is specific to the protein of interest.

Figure 4.7: Western blot of GST vitellogenin fusion proteins using polyclonal rabbit antiserum against *A. vasorum* ES antigens.



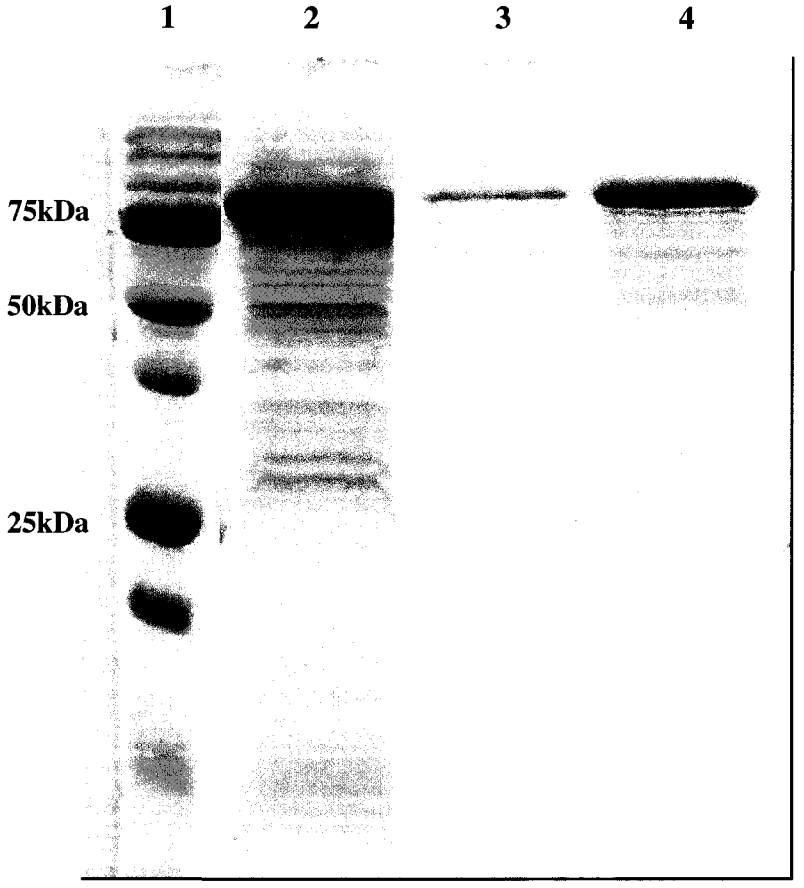
Note: **Lane 1**- Marker, **Lane 2**- negative control (IPTG Induced control GST protein cell lysate), **Lane 3**- GST vitellogenin fusion protein, (IPTG Induced GST fusion protein cell lysate), **Lane 4**- negative control after elution, **Lane 5**- GST vitellogenin fusion protein after elution.
The absence of bands in lane 4 indicates that the ES rabbit polyclonal antiserum did not react with the GST, demonstrating that it is specific to the protein of interest.

Figure 4.8: SDS-PAGE of GST vitellogenin fusion proteins after thrombin cleavage and elution.



Note: **Lane 1**- Marker, **Lane 2**- IPTG induced GST vitellogenin fusion protein cell lysate before purification and elution, **Lane 3 and 4**- thrombin cleavage of fusion protein following elution, **Lane 5**- glutathione beads after thrombin cleavage and elution indicating cleaved product remains adherent to beads

Figure 4.9: SDS-PAGE gel of cell lysates mixed with sepharose beads.



Note: **Lane 1**- Marker, **Lane 2**- induced GST vitellogenin fusion protein cell lysate before purification and elution, **Lane 3**- GST vitellogenin fusion protein after elution, **Lane 4**- sepharose beads on the SDS gel following elutio, the beads still containing the GST vitellogenin protein and this can be seen by the 77 kDa band (the size of the GST vitellogenin fusion protein)

CHAPTER 5: GENERAL DISCUSSION

The purpose of this thesis was to develop a more reliable diagnostic test to detect infection of *Angiostrongylus vasorum* in dogs. *Angiostrongylus vasorum*, also known as the French heartworm, is a metastrongylid nematode that infects the pulmonary arteries and right ventricle of wild and domestic canids. Infections in dogs tend to be chronic, lasting from months to years, and range from subclinical to fatal cardiopulmonary disease (Bolt *et al.*, 1994). The prepatent period of the parasite is usually from 38-57 days (Bolt *et al.*, 1994) and Cury *et al.* (1996) also showed in an experimental study that the range of the prepatent period could be 28-100 days. The lifespan of an adult *A. vasorum* can be as long as its infected host (Conboy, 2004).

Angiostrongylus vasorum infection is diagnosed by detection of first-stage larvae using the Baermann technique or fecal flotation; Baermann examination is considered the technique of choice (Conboy, 2004). However, multiple fecal samples may be required as shedding of the larvae can be intermittent (Oliveira-Junior *et al.*, 2006; Patteson *et al.*, 1993).

The current study was an attempt to develop a serological test, similar to the ELISA test used for the diagnosis of the North American heartworm *D. immitis* (Grieve *et al.*, 1981; Weil *et al.*, 1985), to detect infection with the French heartworm. The ELISA test is capable of quickly detecting either antibodies that have been developed against the parasite or antigens released from the parasite. Infection with *Angiostrongylus vasorum* induces a host antibody response that can be detected by ELISA. This methodology may allow diagnosis prior to the onset of patency and would not be affected by intermittent fecal larval shedding. The indirect ELISA in this study

was used to detect circulating antibodies of *Angiostrongylus vasorum* infection in dogs. However, the indirect ELISA was considered unreliable due to lack of specificity. The indirect ELISA could not differentiate between *A. vasorum* and *Crenosoma vulpis* infection. An additional potential drawback to a test based on detection of host humoral response has been persistent titers after previous exposure, as observed with the serologic tests used to detect *D. immitis* infection in cats (Datz, 2003). This led us to develop the sandwich ELISA test to detect circulating antigens of *Angiostrongylus vasorum* infection in dogs. Adult *A. vasorum* release proteins into the circulation that can act as antigens and be detected by sandwich ELISA. In this version of the ELISA, a microtiter plate is first coated with an antibody specific to the antigen(s) of interest and this same antigen present in the dog serum is detected by the use of another enzyme-labeled antibody specific to the antigen of interest. The *Dirofilaria* antigen test, on which the test developed in this study is based, is used routinely in both diagnostic laboratories and private practices for early diagnosis of heartworm infection (Courtney and Zeng, 2001; Datz, 2003).

The sandwich ELISA test using the rabbit polyclonal antisera had a specificity of 100% and a sensitivity of 92% at an optical density cut-off value of 0.19. Furthermore, the test detected antigens in the sera from 10/10 experimentally infected dogs by 4 weeks post-infection (5 weeks earlier than detection of fecal larval shedding was possible using the Baermann technique). In addition, the test did not cross-react on 30 sera samples from dogs infected with the lungworm, *Crenosoma vulpis*.

In the survey of dogs with signs of cardiopulmonary disease in Newfoundland from 1998-2000, 26% (22/182) were positive for *A. vasorum* on the ELISA compared to

2.7% (5/182) positive on the Baermann test. According to the low kappa statistic (0.34) there was poor agreement between the tests due to the low number of positives on the Baermann test compared to the ELISA. The second fecal survey of dogs with signs of cardiopulmonary disease between 2000-2002 in Newfoundland indicated that 40% (23/57) were positive on ELISA compared to 33% (19/57) positive on the Baermann test. According to the higher kappa statistic (0.70) there was better agreement between the tests. The discrepancy in the kappa statistic comparison of the tests between the two surveys may indicate lower infection intensity was present in the dogs in the first survey. If such were the case, the larval shedding levels may have been lower and more erratic. This would have negatively affected the Baermann technique sensitivity. Furthermore, both sandwich ELISA and fecal testing indicate marked increase in detection of *A. vasorum* in the study population comparing 1998-2000 to 2000-2002. The sandwich ELISA failed to detect 2 dogs that were positive for *A. vasorum* on the Baermann technique. False negatives results can occur with the *Dirofilaria* antigen tests in cases where there are very low parasite burdens (Nelson *et al.*, 2005). Low worm burdens may have played a role in the false negative results on these 2 dogs.

To standardize the test, to increase sensitivity and develop a dependable source of diagnostic antigen(s), we attempted to characterize the antigen(s) detected in the sandwich ELISA by using molecular biology techniques. There is currently no DNA sequence information available for *Angiostrongylus vasorum*. To obtain relevant DNA sequence or at least parts of the DNA, a cDNA library was chosen as the most appropriate way to identify *A. vasorum* proteins. By using a cDNA library vector capable of expressing *A. vasorum* fragments it contained, immunoreactive clones were

identified by screening with a polyclonal antibody from rabbits immunized with *A. vasorum* antigen. This study focused on developing a cDNA library using poly (A) RNA from adult female and male *A. vasorum* to identify immunoreactive clones and screening those clones with the same polyclonal antibody that was used in the sandwich ELISA. Screening of the cDNA amplified library revealed 8 immunoreactive clones. The immunoreactive clones were amplified by PCR using standard T3 and T7 primers, and the PCR products were sequenced. Seven of the 8 sequences coded for 3 proteins – vitellogenin, trypomyosin and heat shock protein70 (Hsp70) that shared homology to other similar proteins in other nematodes. One unique immunoreactive clone from the *A. vasorum* cDNA library did not have homologues in GenBank.

Yolk proteins in nematodes are synthesized in the intestine, secreted from the intestine into the body cavity, and taken up from the body cavity by the gonad to reach oocytes (Sharrock, 1983). From the cDNA library in this study, 4 *A. vasorum* immunoreactive clones had 76% similarity to vitellogenin protein when compared to the nematode *C. elegans*. Of these 4 clones, 3 had overall overlapping base pairs of 2065 while the fourth clone that was isolated was from a different part of the same protein with 1618 bp. Vitellogenin is essential for the parasite's development and reproduction and, therefore, is a potential good candidate for detecting the parasite's presence in infected animals. As rabbit polyclonal antiserum detected the protein in the *A. vasorum* cDNA library screening, it is potentially antigenic during infection, which makes it possible to develop polyclonal or monoclonal antibodies against vitellogenin for diagnostic purposes. Sera from dogs infected with *A. vasorum* could then be identified with the specific antibodies using the sandwich ELISA.

Tropomyosin was the second protein to be identified from cDNA library immunoreactive clones with 889 bp and 97% similarity to tropomyosin protein from *Onchocerca volvulus*, *Trichinella spiralis* and *Trichostrongylus colubriformis*. Tropomyosin occurs in the muscle tissues of the nematodes and is essential for their motility and in *C. elegans* playing a role in ovarian contraction (Ono and Ono, 2004). It was also found in spermatids and spermatozoa of the nematode *Heligmosomoides polygyrus*, an intestinal parasite of woodmice and other rodents (Mansir and Justine, 1996). Like vitellogenin, tropomyosin is an essential protein for nematodes in all stages of development and is antigenic during infection. Hence its antigenic properties and use in immunodiagnostic tools should be also assessed.

Another protein that was identified as an immunoreactive clone from the *A. vasorum* cDNA library was Hsp70. Two clones overlapped each other with an overall of 1939 bp and had 95% similarity to Hsp70 from the nematodes *Caenorhabditis elegans*, *Parastrongyloides trichosuri* and *Wuchereria bancrofti*. The Hsp70s play an important part in parasitic nematodes during host invasion. Nematodes need to tolerate the sudden changes in temperature due to their life cycle, i.e. transferring from cold blooded gastropods to warm blooded mammals (Maresca and Carratu, 1992). Therefore, Hsp70s are expressed at all developmental stages and possess parasite-specific antigenicity. Hence, Hsp70 might also be a good candidate as a diagnostic tool (Bannai *et al.*, 2003; Dobbin *et al.*, 2002).

The fourth immunoreactive clone had a unique DNA sequence and had no homology in the GenBank database, making it potentially unique to *A. vasorum*. Further

protein characterization research on *A. vasorum* and other parasites, may uncover homology in the GenBank database in the future.

Protein purification and expression of these specific segments of antigens will allow the production of specific antibodies targeting the specific antigens. Using these antibodies in the sandwich ELISA prepared against the specific parasite protein(s) released in the circulation of the infected dogs should lead to greater sensitivity and test repeatability than that currently achieved with the whole worm crude antigen polyclonal serum. To obtain a pure protein that could be used as an antigen in this study, we used one of the vitellogenin immunoreactive clones. This protein sequence was chosen as it was the most common occurring sequence in the cDNA library.

For production of a purified protein we used the Glutathione S-transferase (GST) system. In this study, the particular vitellogenin fragment that was used may be especially compact and difficult to elute or, since it is a yolk protein, may be unusually inclined to stick to the glutathione beads because of their sepharose content. The apparent affinity of the protein to the glutathione beads was tested with standard sepharose beads that do not contain glutathione, and the fusion protein did stick to the sepharose beads even when cleaved from GST. To elute the fusion proteins from the glutathione resin, we had to increase the salt concentrations, the pH level, the percentage of Triton X-100, and the molarities of the reduced glutathione buffer in the elution buffer and incubate the fusion proteins at room-temperature. The vitellogenin fusion protein in this study is approximately 77 kDa; it consists of the partial vitellogenin (protein of interest), which is 51 kDa, and the GST, which is 26 kDa. The SDS-PAGE and the Western blot did show a strong, clean band at the 77 kDa.

Other researchers have encountered similar problems in their studies but it did not affect their ability to use GST fusion proteins to immunize mice for developing monoclonal antibodies to such diverse parasites as, *Dictyocaulus viviparous*, *Entamoeba histolytica* and *Schistosoma mansoni* (Abath *et al.*, 1997; Lopez-Monteon *et al.*, 2003; Schnieder, 1992). Therefore, the cleavage conditions appear to depend on the protein of interest and should be optimized for individual proteins with consideration toward protein instability, cleavage efficiency, and purity of the final cleaved protein product.

There is a potential risk for *Angiostrongylus vasorum* to spread from Newfoundland to the rest of Atlantic Canada. Any region containing the biological and climatic factors conducive to the transmission of *Crenosoma vulpis* would also be expected to facilitate the spread of *A. vasorum* as they would share the same reservoir hosts (red foxes) and similar intermediate hosts (gastropods). Given the volume and frequency of travel between Newfoundland and the rest of Atlantic Canada, the introduction of *A. vasorum* to the mainland appears to be inevitable. There have been several reports of *A. vasorum* infected dogs imported into North America that do not appear to have resulted in the establishment of endemic foci (Perry *et al.*, 1991; Williams *et al.*, 1985). Tebb *et al.* (2007) reported a case of *A. vasorum* in Australia in a Cocker Spaniel that was imported from Surrey, England. The dog was quarantined for 4 months upon arriving to Australia and was presented to the veterinary hospital with a cough and oculonasal discharge, lethargy and weight loss. Fecal samples were examined with the Baermann technique and first-stage *A. vasorum* larvae were found. *A. vasorum* is not yet established in Australia. Nonetheless, the distribution of *A. vasorum* in different parts of the world such as South America, North America, Europe,

Turkey and Africa imply that it can potentially spread anywhere. This demonstrates that the ease of travel between countries and regions can permit the introduction of *A. vasorum* to non endemic areas. The distribution of *A. vasorum* may be limited by climate, however. It occurs only in regions of Newfoundland where the mean temperatures do not go below -4°C (Jeffery *et al.*, 2004). *Angiostrongylus vasorum* infection was reported in areas with wet, humid and milder climates (Dodd, 1973), unlike *C. vulpis* infection, which is more common in colder weather (Jeffery *et al.*, 2004). The potential factors, if any, limiting the south/west spread of *A. vasorum* in North America are unknown. The cat lungworm, *Aelurostrongylus abstrusus*, also in the same family Angiostrongylidae has a similar life cycle (gastropod intermediate host) to *A. vasorum*. It occurs throughout the eastern half of North America and therefore this region may also support the spread of *A. vasorum*.

In current practice, fecal diagnostics are not utilized until dogs show clinical signs suggestive of *A. vasorum* infection. As with *D. immitis* infection, angiostrongylosis dogs may be infected for months to years prior to developing clinical disease and at this stage may have suffered severe permanent cardiopulmonary damage. The availability of an accurate, affordable diagnostic method and a move towards routine diagnostic screening in healthy dogs (as is done for *D. immitis*) will allow earlier detection of *A. vasorum* infected animals. The treatment prognosis greatly improves in *D. immitis* infected dogs diagnosed and treated prior to the onset of clinical disease compared to those already showing signs of heart failure (Courtney and Zeng, 2001; Datz, 2003). The same would presumably be true for dogs infected with *A. vasorum*.

For future study, the infection status of the ELISA positive dogs/ Baermann negative dogs needs to be confirmed. Secondly, the parasite burden threshold of detection (how many worms are needed to get an ELISA positive test) and the length of time antigenemia persists after treatment needs to be determined.

A mouse immunized with the protein found in this study could be used for production of monoclonal antibodies that might be effective in the sandwich ELISA. The use of specific monoclonal antibodies could increase the sensitivity of the ELISA used to evaluate *A. vasorum* infection in dogs. Furthermore, production of monoclonal antibodies could provide a more stable and reliable supply of reagents for diagnostic tests, which would allow the sandwich ELISA test to be standardized for everyday usage in the diagnosis of *A. vasorum* infected dogs. Nevertheless, it should be noted that in this study we have partial sequences of the proteins and because monoclonal antibodies target individual epitopes, and we do not have the 5'end (N-terminus) of the vitellogenin gene since we do not have the first methionine codon (ATG) on any of our clones, it may be difficult to know if we have the best monoclonal. On the other hand, a polyclonal antibody to the partial protein may be excellent since several epitopes are included. However, the full protein sequence can be obtained by performing the 5' RACE (rapid amplification of cDNA ends) on all 4 genes described in this study. Besides the diagnostic benefits, identification of *A. vasorum* antigens can be advantageous in the future by allowing researchers to select encoded proteins with potential protective capacities, which may be possible targets for vaccine development.

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