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Creation of an Antemortem Serological Diagnostic Test of *Parelaphostrongylus tenuis*

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To the Graduate Council:

I am submitting herewith a thesis written by Jessie Erin Richards entitled "Creation of an Antemortem Serological Diagnostic Test of *Parelaphostrongylus tenuis*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

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Creation of an Antemortem Serological Diagnostic Test of *Parelaphostrongylus tenuis*

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ABSTRACT

Parelaphostrongylus tenuis is a parasitic nematode common among deer, elk, moose, and horses. The parasites attack the central nervous system, laying their eggs in meningeal tissue resulting in high morbidity and mortality. Techniques currently available for the most accurate diagnosis include necropsy to detect adult worms in the brain and spinal cord. The goal of the present research was to develop a way to accurately diagnose *P. tenuis* infection antemortem. A gene encoding a *P. tenuis* protein was inserted into an expression plasmid and propagated in *E. coli*. The recombinant protein was affinity purified, separated on SDS-PAGE gels and transferred to nitrocellulose. Western blots were utilized to identify anti-*P. tenuis* antibody present in blood, serum and spinal fluid using sera from known positive and negative animals. Enzyme conjugated anti-cervid antibody produced in chickens was used to detect serologically positive elk, moose and deer. After western blots were confirmed to be effective diagnostics, enzyme-linked immunosorbent assays (ELISAs) were used to test overlapping 10-mer synthetic peptides for the development of a more cost effective, less labor-intensive test. However, due to inconsistent results and cross reactivity with other similar organisms, we have moved our attention to a full genomic analysis of *P. tenuis* to further distinguish a more definitive antigen. This effort is ongoing but is expected to identify epitopes that are unique to *P. tenuis* and serve as a useful diagnostic assay.

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CHAPTER ONE

INTRODUCTION AND GENERAL INFORMATION

Introduction to *Parelaphostrongylus tenuis*

Parelaphostrongylus tenuis is a parasitic nematode in the protostrongylid family that is often referred to as brain worm or meningeal worm. It is a long, threadlike worm that can be found in the meninges of white-tailed deer (*Odocoileus virginianus*). Worms are found in the veins and venous sinuses of the cranial meninges as well as free in the cranial subdural space of its definitive host, white-tailed deer where it usually causes little to no disease state.

P. tenuis possesses a complex life cycle where it infects two distinct hosts. *P. tenuis* adults can be found in the deer's meningeal tissue within the veins and venous sinuses. Females lay unembryonated eggs that travel via venous blood to the lungs where they hatch into the first larval stage (L1). L1s then migrate to the alveolar airspace, move up the trachea, are swallowed, and excreted on the surface of fecal pellets after passing unharmed through the digestive tract (Anderson 1963).

A variety of terrestrial gastropods, such as snails or slugs, serve as intermediate hosts (Anderson and Prestwood 1981). They ingest the larva in the excrement or that has been dispersed into the soil. The nematode matures to the infective third larval stage (L3) within the foot tissue of the gastropod. Maturation in the intermediate host takes about four weeks at temperatures between 18° C and 30° C (Anderson 1963). L3s can endure freezing temperatures, enabling them to overwinter in the intermediate host and are

thought to remain infective for the entirety of the intermediate host's life (Lankester and Anderson 1968).

P. tenuis infects white-tailed deer upon accidental ingestion of a gastropod while grazing, thus, completing the cycle. L3s released from the gastropod's tissues will penetrate the abomasal wall and enter the peritoneal cavity (Anderson 1963, Anderson and Strelive 1967). They then are thought to migrate directly to the spinal cord (Anderson and Strelive 1967). The worms leave the spinal cord via dorsal nerve roots and move towards the cranium in the spinal subdural space (Anderson 1968).

Significance of Disease

In accidental hosts such as moose and elk, *P. tenuis* does not migrate to the appropriate meningeal tissue and instead colonizes the nervous tissue in both the brain and spinal cord. This causes significant neurological damage to the animal by both mechanical injury as well as inflammation. Partially due to this aberrant migration, the nematode is unable to complete its life cycle in these atypical, dead-end hosts.

Unfortunately, the most reliable diagnosis of *P. tenuis* in aberrant hosts entails a necropsy in which worms are observed in the brain or spinal cord. Fecal tests such as the Baermann test are not viable as moose and elk do not reliably shed larvae in their feces. Additionally, presumptive diagnosis based on presenting neurological symptoms is also not always reliable. The lack of definitive diagnostics has caused the prevalence of this disease to be difficult to assess and properly treat.

Basis of Research Towards Antemortem Diagnosis

To combat the limitations of current diagnostics, research performed by Duffy, MacAfee et al. (2002) suggests that atypical hosts could be serologically diagnosed by enzyme-linked immunosorbent assay (ELISA) for antibodies that are produced in response to the aspartyl protease inhibitor, *Pt*-API-1. Additionally, studies performed by Ogunremi et al. suggest serological diagnosis of *P. tenuis* involving the development of an ELISA that utilizes the unique 37-kDa antigen present in *P. tenuis* show significant promise (Ogunremi, Lankester et al. 1999b). However, neither of these tests are commercially available.

Cross reactivity, however, is something that is not well discussed in this research and needs further investigation not only regarding *Pt*-API-1, but also any antigen considered for serological diagnosis. The efficacy of this antigen in serological testing and potential for cross-reactivity will be addressed in this thesis.

CHAPTER TWO

LITERATURE REVIEW

Clinical Signs in the Definitive Host

Signs of illness due to infection are rare in the definitive host. Experimental doses of three-hundred L3 stage larvae given to a fawn showed no clinical signs except for a loss of guard hairs. Upon necropsy, sixty-five adult *P. tenuis* nematodes were recovered (Pybus, Samuel et al. 1989). Another study in which white-tailed deer were experimentally infected with two-hundred L3 larvae disclosed no clinical signs throughout the experiment (Anderson 1968).

A couple of accounts have indicated, however, that illness in white-tailed deer is possible in some circumstances. An enormous number of worms were recovered from a pregnant, three-year old doe with an unsteady gait, head tilt, and moderate nystagmus. In this case, eighty adult *P. tenuis* were recovered from the subdural space as well as an estimated twenty to thirty worms within the sinuses (Prestwood 1970). Another case involved a male white-tailed deer which had been experimentally infected with *Haemonchus contortus* larvae for a study. The animal began showing signs of ataxia and started passing *P. tenuis* larvae in its feces twelve days after the start of the experiment. The animal died at day sixteen of the experiment and ten live adult *P. tenuis* were collected at necropsy from the subdural space. Significant subarachnoid and subcortical curvilinear hemorrhages were observed over the cerebrum as it appeared the parasite had invaded these spaces (Eckroade, Zu Rhein et al. 1970).

These cases of significant clinical disease in white-tailed deer are not the norm. For the most part, mature worms will be found in the epidural space or only partially imbedded in the dura (Anderson 1963). It is in these locations that they are least invasive and induce minimal inflammation in the definitive host.

Prevalence and Intensity in the Definitive Host

Dependent on geographical location, most white-tailed deer are infected with *P. tenuis* at some point in their life. A study done by Karns (1967) found that forty-nine percent of one-hundred forty deer in Minnesota examined were infected with *P. tenuis.* In central Minnesota, where population density of the host was greatest, prevalence was significantly higher than the lower density regions, suggesting prevalence of infection is host density dependent. Similarly, Behrend (1968) showed that eighty-four percent of one-hundred ninety six white-tailed deer in all counties of Maine contained adult nematodes and places with high density of white-tailed deer had higher incidences of infection. Other studies, however, indicate that density of host populations does not increase incidence of infection and therefore had no significant correlation (Gilbert 1973, Bogaczyk, Krohn et al. 1993).

Underestimations of prevalence is thought to be a significant problem with this parasite and could account for the disagreement about the correlation between deer density and incidence of infection. Underestimations, in part, are due to the nature of definitive diagnosis. Worms within venous sinuses are easily missed during necropsy, especially if the head is damaged to the point that it cannot be properly examined. Fecal examination for prevalence is also often misleading as evidence shows that adult white-

tailed deer acquire few additional worms after their first or second summer, creating the possibility of non-productive unisex infections (Slomke, Lankester et al. 1995). Similarly, A study done Duffy, Greaves et al. (2002) suggests that infection is also long-term with the possibility of long latent periods. This highlights the necessity for an antemortem diagnosis for *P. tenuis* other than fecal exam to identify non-patent infections and avoid introducing infected animals to non-endemic areas.

In one study performed by Comer et al. (1991) prevalence in the southeastern United States was studied. *P. tenuis* was found commonly within the white-tailed deer of Arkansas, Kentucky, Louisiana, Maryland, North Carolina, Tennessee, Virginia, and West Virginia as well as the norther portions of Alabama and Georgia. In this study, 95% of the time the worm was found within the first five deer examined. No infected deer were found in Florida since the discovery of a single worm in 1968.

Impact and Prevalence on Atypical Hosts

In atypical or aberrant host, *P. tenuis* causes significant clinical disease. Severity of infection in atypical hosts is thought to be multifactorial. The proportion of worms that reach the central nervous system is higher and longer development in the spinal cord results in a larger size and coiling behavior. Additionally, they frequently invade the ependymal canal (Anderson 1968).

P. tenuis is the causative agent of "moose sickness," a neurologic disorder by locomotor dysfunction, fearlessness, circling, weakness, impaired vision, deafness, head-tilt, and paraplegia (Anderson 1964). Disease is apparent in moose with as little as one

infecting worm (Lankester 2001). Peterson (1989) reported the presence of abnormal antlers and kidney stones in moose displaying signs of parelaphostrongylosis. Occasionally, it is possible for moose to show little to no clinical signs during infections (Gilbert 1974, Thomas and Dodds 1988), making it feasible for moose to coexist with deer in endemic regions.

Experimental inoculation of moose has shown that large doses of two-hundred L3 larvae given to moose calves at 1 month prove fatal with debilitating neurological signs. These large doses result in heavy infections with up to forty-one worms recovered from the central nervous system of one animal (Anderson 1964). These doses, however, are not realistic as the intermediate host rarely carriers more than a few L3 larvae with an overall prevalence in gastropods being only 0.01% to 0.8% where moose and white-tailed deer coexist (Lankester 2001). Lankester (2002) performed an experiment in which a low dose of three to ten L3 larvae were given to moose calves. Each calf developed neurological signs twenty to twenty-eight days post inoculation, but all had recovered by day one-hundred thirty-one. A second, larger dose of fifteen worms was then administered to the calves and no clinical signs developed. Positive control calves that were given the larger dose initially developed non-resolving neurological signs and had to be euthanized. This indicated that low doses in moose can elicit a degree of immune protection against future infection and implicates a possibility of vaccine development in atypical hosts.

Moose sickness is theorized to be a contributing factor to moose decline, but little is certain about its impact. Since, the disease first described in 1932 (Wallace, Thomas et

al. 1932), nearly five-hundred cases had been reported in the literature by 1994. Furthermore, moose populations tend to decrease with the increased population density of white-tailed deer and incidence of infection (Whitlaw and Lankester 1994). As expected, the incidence of disease in moose has positive correlation with white-tailed deer populations (Gilbert 1974, Dumont and Crete 1996). Research has shown that moose populations can persist in *P. tenuis* endemic regions as long as the white-tailed deer population remains below 6/km^2 (Whitlaw and Lankester 1994).

While moose sickness has been widely reported, few studies provide estimates of *P. tenuis* in moose populations. Over a four-year period in Maine, 80% of one-hundred fifty-three moose that were showing clinical signs were diagnosed with parelaphostrongylosis at necropsy. Additionally 25% killed by poachers and 10% to 15% killed by vehicles or other miscellaneous accidents were infected (Gilbert 1974).

Minnesota also has reported *P. tenuis* prevalence in cervids. Fifty-four carcasses of moose that died of unknown causes or were euthanized due to perceived illness between 2003 and 2013 were submitted for necropsy along with eight moose that had died from vehicular accidents between 2009 and 2013. Signs of neural migration attributed to *P. tenuis* was found in 45% of these moose (Wuenschmann, Armien et al. 2015).

Reintroduced elk in Tennessee's Cumberland Mountains from Alberta have also suffered mortality due to *P. tenuis* infection (Kindall et al., 2011). Of the 62 mortalities, 26 were unable to be determined. However, of the remaining 36 cases, 5 were attributed to disease caused by *P. tenuis.* They also predicted an additional 5 deaths with *P. tenuis*

as a contributing factor. At this time, the threat of meningeal worm to elk may play a role in the failure of sustained reintroduction to this region.

Parelaphostrongylosis is also a significant problem in domestic animals such as llamas, sheep, goats, and alpacas and causes monetary loss to farmers. It is also a concern for zoos and game farms and fear of spreading the disease has led to restrictions on the translocation white-tailed deer (Lankester 2001).

Population Distribution of Hosts and Their Interactions

Prior to the early 1900s, moose and deer populations had little overlap and therefore, the two species had limited interactions and potential for transmission of diseases (Peterson 1955). The expansion of white-tailed deer into moose ranges is attributed to logging in the early 1900s (Anderson 1972). An increase in deer population has led to a decline in moose numbers in these overlapping areas as well as many moose in these areas presenting with moose sickness (Anderson and Lankester 1974). With nothing more than anecdotal and opportunistic sightings of sick animals, it's difficult to accurately estimate the impact of the disease on the moose population.

Population overlap has also caused issues in other cervid species. In the eastern United States, *P. tenuis* is one of the most common diseases in restored elk (Raskevitz, Kocan et al. 1991). In 2011, the Missouri Department of Conservation began efforts to restore elk in southern Missouri and later research found through necropsy that up to 33% of mortality could be attributed to meningeal worm (Chitwood, Keller et al. 2018).

http://www.jwildlifedis.org/doi/abs/10.7589/0090-3558-27.2.348?code=wdas-site

Diagnosis of Parelaphostrongylosis

Presently, the only way to definitively diagnose *P. tenuis*, is the recovery of adult worms from the central nervous system of infected animals or detection by PCR of neural tissue. This is made difficult by the skill and care it takes to located worms within the venous sinuses and the fact that a single worm can cause devastating neurological effects in an atypical host. Clinical signs in susceptible species that are thought to have been in contact with white-tailed deer or the gastropod intermediate host are suggestive of parelaphostrongylosis and is the only antemortem diagnostic employed by veterinarians.

Fecal diagnostics for both the definitive host and aberrant hosts have significant limitations. The traditional fecal examination using the Baerman technique may only recover a fraction of the larvae present and has poor repeatability. Up to 67% of the larvae may remained lodged on the sloping surface of the glass, severely underrepresenting the true larval load (Forrester and Lankester 1997). Furthermore, *P. tenuis* is one of the many dorsal-spined larvae found in cervid feces and the morphology of these closely related species is very similar, (Pybus and Shave 1984). Also, in the case of unisexual infection, deer would not pass larvae at all. Conversely, false positive diagnosis has also been shown to be a risk of fecal tests. Duffy, Keppie et al. (1999) demonstrated that stage-one larvae ingested by rats pass through the alimentary tract unharmed. This suggests the possibility of susceptible hosts accidentally ingesting larvae at this stage and uninfected hosts passing larvae and misdiagnosed as positive, reinforcing the need for a diagnostic that accurately reflects the host's infection status.

Limitations are even more pronounced in aberrant hosts such as moose and elk. Welch, Pybus et al. (1991) found that most infected elk in their study shed meningeal worm larvae intermittently and at low densities if they did so at all. In moose, a study by Karns (1977) showed that only 29% of moose diagnosed as sick were passing larvae. Additionally, moose frequently pass *Parelaphostrongylus andersoni* and *Elaphostrongylus rangiferi* in their feces. These are similar species of nematodes with dorsal-spined larvae that cannot be reliably differentiated morphologically from *P. tenuis* (Lankester and Fong 1998). Differentiation of these larvae can, however, be accomplished by polymerase chain reaction (PCR) (Gajadhar, Steeves-Gurnsey et al., 2000).

Blood chemistry and hematology are often used in conjunction with the presence of clinical signs to diagnose parelaphostrongylosis. In an endemic area, llamas had been diagnosed based on clinical signs and eosinophilic pleocytosis (Lunn and Hinchcliff 1989). Two llamas in another study were experimentally infected with *P. tenuis,* one with forty-eight worms and the other with six. Each developed clinical signs along with cerebrospinal fluid (CSF) eosinophilia (Foreyt, Rickard et al. 1991). Furthermore, a study by Pinn, Bender et al. (2013) investigated the accuracy of CSF eosinophil percentage in the diagnosis of parelaphostrongylosis. It was found that more than 17% of eosinophils in CSF had a sensitivity of 85% and specificity of 92% for *P. tenuis* infection.

However, it is important to note that previous research by Rickard, Smith et al. (1994) have shown that experimental infections yielded no abnormal blood chemistries. Additionally, Dew, Bowman et al. (1992) asserts similar doubts exists for this diagnostic

in goats as well as white-tailed deer which argues that there is some degree of variability in these numbers.

Many of the issues with fecal diagnosis and blood chemistry can be overcome using molecular techniques. Gajadhar, Steeves-Gurnsey et al. (2000), has made progress with the creation of a PCR to diagnose and differentiate between the morphologically similar dorsal-spined larvae of the protostrongylid subfamily, Elaphostrongylinae. The designed primers selectively amplify DNA of the internal transcribed spacer 2 (ITS-2) region between 5.8S and 28S ribosomal genes. This can differentiate between larvae of the *Elaphostrongylus* and *Parelaphostrongylus* genera based on fragment size. Additionally, one primer set in a single PCR assay was successfully able to differentiate between species within the genera *Parelaphostrongylus*: *P. tenuis, P. odocoilei,* and *P. andersoni.*

Serological Diagnosis of *Parelaphostrongylus tenuis*

Focus has since shifted to a serological diagnosis using immunological and molecular techniques. Finding that blood chemistry offered little support in diagnosis of parelaphostrongylosis, Dew, Bowman et al. (1992) demonstrated the presence of antibodies in serum and CSF of two goats to antigen extracts of adult *P. tenuis* using an enzyme-linked immunosorbent assay (ELISA). They also demonstrated antibodies in two white-tailed fawns, but only in the CSF. Not long after, Duffy, Tanner et al. (1993) used similar techniques to detect serum antibodies in fawns, both experimentally and naturally infected.

Neumann, Pon et al. (1994) immunized rabbits with *P. tenuis* soluble extracts and identified nine somatic antigens via immunoblot using the acquired rabbit-anti-*P. tenuis* immunoglobulin G (IgG). Two of these antigens belonged to the infective third-stage larvae and measured 25-30 kD and 13 kD. The remaining seven were adult somatic antigens, four measuring 170-120 kD, two 55 kD, and another 13 kD antigen. These antigens distinguished *P. tenuis* from two other closely related nematodes, *Dictylocaulus viviparus* and *Trichinella spiralis*. It was later found by Bienek, Neumann et al. (1998) that elk experimentally given large doses of *P. tenuis* recognized both larval and adult antigens, but small doses only elicited a response to larval antigens. Further experiments demonstrated elk with adult worms in the central nervous system recognized 25-27 kD, 28-30 kD, and 34-36 larval antigens, but none of these were consistently recognized at the more realistic, lower doses. Unfortunately, however, these experiments also called attention to anti-*P. tenuis* antibodies displaying cross-reactivity with *D. viviparus* antigens, despite Neumann's previous findings.

Ogunremi, Lankester et al. (1999a) evaluated excretory-secretory and somatic worm antigens for use in serodiagnosis of experimental *P. tenuis* infections of white-tailed deer. The three antigen preparations explored were the excretory-secretory products of third-stage larvae (ES-L3), the somatic antigens of third-stage larvae (sL3), and the somatic antigens of the adults stage (sA). The larval antigens (ES-L3 and sL3) were both consistently present and reliable for the early detection of *P. tenuis* while no anti-sA IgG was ever detected. Unfortunately, cross-reactivity continued to be an issue

and all three antigen preparations had significant cross reactivity with related parasites, namely *P. andersoni, E. rangiferi,* and *E. cervi.*

It was at this point that an unique 37-kD antigen present in all three antigen preparations was identified (Ogunremi, Lankester et al. 1999b). Serum from infected white-tailed deer reacted with five distinct third-stage larval antigens of 105-kD, 45-kD, 37-kD, 32-kD, and 19-kD, but only the 37-kD antigen did not cross react with serum from caribou infected with *P. andersoni* and *E. rangiferi*. It is possible that this antigen is the same as the 36-kD antigen isolated previously by Neumann, Pon et al. (1994). Since then, experiments using the excretory secretory products of third-stage larvae for immunological diagnosis has shown promise in both moose and elk (Ogunremi, Lankester et al. 2002).

Not long after expressing the need for an antemortem serological diagnosis for *P. tenuis* due to false positives in fecal samples and the establishment of the nematode as a long-lived parasite in white-tailed deer, Duffy and Burt (2002) began investigating the excretory-secretory antigens of adult *P. tenuis* for immunodiagnostics. A single 42-43-kD protein was consistently recognized by sera of red deer infected with members of the elaphostrongyline family, *P. tenuis, P. andersoni,* or *E. cervi,* but not recognized by control deer infected with *V. sagittatus,* a related protostrongylid. The need for a specific, *P. tenuis* immunodiagnostic utilizing adult antigens, however, was still evident.

Discovery of an Aspartyl Protease Inhibitor

Using mRNA from adult *P. tenuis*, Duffy, MacAfee et al. (2002) constructed a cDNA library to identify proteins of potential significance. The library was screened with sera from infected red deer and immunized Albino Oxford (AO) rats, a protein was found that, after a BLASTp search, resembled aspartyl protease inhibitors from several other parasitic nematodes, namely *Onchocerca volvulus, Ostertagia ostertagi, Caenorhabditis elegans, Dirofilaria immitis,* and *Acanthocheilonema viteae.* To deduce a full-length reading frame, consensus nucleotide sequences from orthologous expressed sequence tags of *Ascaris suum, Brugia malayi,* and *Parastrongloides tricosuri* were used. An additional orthologue from *Ascaris suum* was then identified by a BLASTp search that showed 19% identity to *P. tenuis.* Highest consensus in sequence identity was with an orthologue from a similar nematode, *Ostertagia ostertagi,* at 74%. This gene was dubbed *Pt*-API-1 with a predicted mass of the mature protein of approximately 23.4-kD.

The native protein was identified in excretory-secretory products by affinity-purified deer antibodies to r*Pt*-API-1. They bound to a protein of 27-kD. Rat anti-r*Pt*-API-1 antibodies also bound to a 27-kD protein when probed with HRP-conjugated goat anti-rat IgG on a Western blot. Additionally, pooled sera from infected red deer and rat antibodies to excretory-secretory antigens also bound to a 27-kD antigen assumed to be *Pt*-API-1, implicating a role of this protein in interaction with the host. The mass of the protein was estimated to be 33-kD when resolved under reducing conditions by SDS-PAGE.

Immunohistochemistry was performed on adult male and female nematodes as well as first and third-stage larvae for detection of *Pt*-API-1. The adults, as well as a slug containing third-stage larvae, were embedded in tissue freezing medium. First-stage larvae were extracted from feces and embedded in tissue freezing medium within glycerin capsules. Cross-sections were treated with rat anti-r*Pt*-API-1 that detected the native antigen in the gonad, muscle, and on the cuticle of the adult organisms. The native protein was also detected within internal structures of third and first-stage larvae. The large presence observed in female gonads implicated release during egg-laying and presence on the cuticle could account for the excretory-secretory component of the protein.

Continued experimentation included PCR on adult, third-stage, and first-stage *P. tenuis* using primers for *Pt-api-1* and a ubiquitous gene from heat shock protein-70 (HSP-70). Transcription for both genes was confirmed in all organisms further demonstrating the presence of this protein in each examined life-stage.

Red deer were experimentally infected with either ten or twenty-five infective third-stage larvae. ELISAs performed on sera from the deer detected infected animals twenty-eight to fifty-six days post inoculation. At necropsy, *P. tenuis* adults were recovered from eleven of the twelve infected red deer. During the experiment, patent periods in red deer were observed with detection of L1 larvae in fecal samples. In animals that expressed patent periods, antibodies to *Pt*-API-1 persisted for up to two years. However, patent periods had no correlation with level of antibody response, nor did degree of exposure. Secondary inoculations with equivalent doses were given at

various intervals, but none of these secondary inoculations yielded reestablishment of infection, suggesting an acquired immunity in red deer.

Aspartyl Protease Inhibitors in Other Parasites

Protease inhibitors are utilized by a variety of helminths to survive within the host. Specifically, aspartyl protease inhibitors have been identified in *A. suum* (Martzen, McMullen et al. 1990), *O. ostertagi* (De Maere, Vercauteren et al. 2005), *T. columbriformis* (Shaw, McNeill et al. 2003), *O. volvulus* (Chandrashekar, Ogunrinade et al. 1993), *D. immitis* (Hong, Mejia et al. 1996), *P. tenuis* (Duffy, MacAfee et al. 2002), *A. viteae* (Willenbucher, Hofle et al. 1993), *B. malayi* (Dissanayake, Xu et al. 1993), *A. caninum* (Delaney, Williamson et al. 2005), and *H. contortus* (Li, Gadahi et al. 2017).

As Duffy, MacAfee et al. (2002) acknowledged, these aspartyl protease inhibitors are highly immunogenic proteins in several other previously studied nematode species, namely *B. malayi, O. volvulus,* and *Dirofilaria immitis*. Antibodies against Bm33 of *B. malayi* were observed in infected-patients (Dissanayake, Xu et al. 1993). Similarly, Tume, Ngu et al. (1997) used sera from four-hundred forty-one patients to test the efficacy of the aspartyl protease inhibitor Ov33 from *O. volvulus* in diagnostics. Using two recombinant proteins, the combined sensitivity of the assay was 94%. Using the same protein, Lucius, Erondu et al. (1988) found similar results with sera from 96% of patients with onchocerciasis recognizing this antigen, but not similar antigens from *B. malayi* or *D. immitis.* Another Ov33 homologue, DiT33, from *D. immitis* was used to

detect antibodies in infected dogs and cats (Mejia, Nkenfou et al. 1994, Hong, Mejia et al. 1996).

De Maere, Vercauteren et al. (2002) identified protective antigens of *O. ostertagi* with antibody probes from calves immunized with multiple infections and from primary infected animals. western blots of extracts and excretion-secretion material from third-stage, fourth-stage, and adult parasites to identify antigens that were recognized by the screening antibodies. One of the antigens identified was a 28-kD aspartyl protease inhibitor that was exclusively recognized by the antibodies from the immunized calves. In a later study, the antigen was shown to share an 82% homology to a *T. colubriformis* aspartyl protease inhibitor. It was also shown that this protein was expressed in all life-stages in western blots and RT-PCR (De Maere, Vercauteren et al. 2005).

Due to their proven immunogenicity in a variety of helminths, aspartyl protease inhibitors are an excellent target for serological diagnosis. However, this does raise concerns about cross reactivity between nematodes. While some research has been done to address these cross-reactivities, there is still some room for investigation. If it were to be used in *P. tenuis* diagnostics, the antigen would need to be distinct from other common nematodes.

CHAPTER THREE

MATERIALS AND METHODS

Production of the *Pt*-API-1 Protein

The *P. tenuis* aspartyl protease inhibitor (*Pt*-API-1) gene was optimized for codon usage in *E. coli* and produced as a synthetic gene. The *Pt*-API-1 gene was cloned into an expression vector and *Pt*-API-1 protein was expressed in BL21 (DE3) competent *E. coli* grown in LB ampicillin broth. Protein expression was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells from overnight culture were harvested by centrifugation at 10,000g for 15 minutes at 4°C. Protein was extracted using bug buster extraction reagent and protease inhibitor was added to ensure protein integrity. After a 20-minute incubation period, insoluble debris was removed by centrifugation at 10,000g for 30 minutes at 4°C and supernatant was loaded onto resin for polyhistidine-tagged purification. Three elutions were taken and Bradford assay was performed to assess level of protein in the elutions.

Western Blot Diagnosis

In 2015, western blots were utilized to identify the presence of anti-*P. tenuis* API antibody in the sera of known infected or uninfected deer. Eluted protein was run on SDS-PAGE where it could be identified in Coomassie Blue or western immunoblots as the band between the 28 and 38 kD marker as indicated by Duffy et.al. The protein from the polyacrylamide gel was transferred to nitrocellulose membrane via the wet blot technique. The membrane was cut into strips and then individually treated with primary

antibody in the form of sera of both known infected and uninfected cervids. It was then treated with HRP conjugated anti-cervid secondary antibody produced in chickens. Visualization of the bands was achieved with 4-chloro-1-naphthol which produces an insoluble purple product. The presence of a band at the 28-38 kD region indicated the presence or absence of anti-*P. tenuis* antibodies present in the selected sera. This test was successful enough to warrant tentative diagnostics to be run on elk sera collected from Maine, New York, Minnesota, Pennsylvania, New Hampshire, and Tennessee where they already suspected *P. tenuis*.

Testing with Synthetic Overlapping Peptides

Enzyme linked immunosorbent assay (ELISA) was utilized to test overlapping 10-mer synthetic peptides to further identify unique epitopes of the API protein. We used known positive and negative serum samples which were confirmed by Western Blot to compare potential points of cross-reactivity along the antigen. This test also used the same enzyme conjugated anti-cervid antibody as a secondary antibody. 3,3',5,5'-tetramethylbenzidine (TMB) substrate followed by a stop solution of 0.18 molar sulfuric acid (H2SO4) was utilized to quantify antibody concentration via optical density.

The first set of peptides tested covered the entire *Pt*-API-1 protein and consisted of 29 overlapping sequences. All peptides were probed with positive sera to identify peptides that represented epitopes with high immunogenicity. These identified peptides were further analyzed and probed with negative sera to assess potential for false positives.

An additional set of peptides was subsequently used in an attempt to improve the quality of the assay. These new peptides (1-21) were overlapping peptides of an epitope of interest from the *P. tenuis* API protein identified previously. Peptides 1 and 13 were excluded from the plate due to size constraints and had been previously identified as not useful. The overlapping nature of these peptides also made such exclusions inconsequential. Peptides 22, 23, and 24 from this same group were from *O. ostertagi*, *D. viviparus,* and *H. contortus* respectively and represented the sequence of amino acids found in the same epitope of those species. These were expected to be antigenically similar to our *P. tenuis* protein and therefore were included to assess cross-reactivity.

To further assess cross-reactivity and false positives, sera from a negative sample that consistently gave false positives on Western Blot and previous ELISAs was pretreated with peptides 22, 23, and 24 separately to investigate whether pretreatment with cross-reactive antigens would be effective at absorbing cross reactive antibodies to improve the specificity of the test. The pretreated serum was then coated on plates of peptides that had been shown to have the highest incidence of cross-reactivity (peptides 4, 5, and 6).

To confirm that reactions were not simply instances of antibodies binding to the plates themselves, a dot blot was performed on nitrocellulose membrane separated into a 6x8 grid. All 24 peptides were dotted on the membrane and allowed to dry. The remaining 24 grid squares were treated directly with positive serum samples (see Figure 3.1). The blot was blocked for 30 minutes in PBS-Tween and then incubated with a 1:100 dilution of all positive banked sera samples (1-25) for 30 minutes. It was washed

three times with PBS-Tween for 5 minutes each before treatment with 1:500 dilution of chicken anti-cervid HRP secondary antibody. It was washed again three times and, as before, treated with 4-chloro-1-napthol.

Genomic Analysis for Investigation of Novel Antigens

Full genomic sequencing was done on *P. tenuis* adult worms harvested from dead white-tailed deer from a hunter check station in Oak Ridge, Tennessee. DNA purification was achieved with the MasterPure DNA Purification kit. A Nextera DNA Library Prep kit was then used to prepare the samples for sequencing after quality was ensured on a bioanalyzer. In lieu of the Zymo cleanup step, we used a Min-Elute PCR Purification kit for cleanup of tagmented DNA. The libraries were sent to the UT Genomics Core Facility for sequencing using Illumina technology and are presently being analyzed. Parasitic nematode antigens have already been identified and will be used for further investigation of unique antigen epitopes.

In addition to DNA, RNA libraries were also prepared. Nematodes were harvested from the brains of dead white-tailed deer from the same hunter check station and stored in RNA later at -20° C. The RNA was purified using the MasterPure RNA Purification kit and then the library was prepared using the Illumina Tru-seq RNA-seq protocol.

Sequencing was performed on an Illumina MiSeq at the University of Tennessee Genomics Core. It was loaded at 6 picomolar with 5% 6 picomolar phiX on a version 3 flow cell reading 250 bases, paired end.

Blast2Go software was then used to further identify proteins from transcriptomic data (Gotz et al, 2008). Results came from running a BLASTx that utilized a public NCBI Blast service (QBlast). The nematode (taxa: 6231, Nematoda) taxonomy filter was used to increase the speed of results obtained and limit useless matches. The BLAST expectation value was set at 1.0E-3 which was the default statistical significance threshold. InterPro analysis was performed using Cloud IPS databases to further aid in identification of proteins. Mapping and annotation were also utilized for completeness and further descriptions to help deduce the function of hypothetical proteins.

Further investigation of transcriptome was performed using the KEGG Orthology and Links Annotation (KOALA) service (Kanehisa, M et al, 2016). A BlastKOALA was performed on 892 sequences that were able to be translated in Blast2Go. The taxonomy filter for nematodes was applied to the search. Of these sequences, 518 entries (58.1%) were annotated and grouped into functional categories. Additionally, a GhostKOALA job was also performed resulting in 540 (60.5%) annotations. This provided statistics that indicated taxonomic category for the sequences.

P. tenuis Protein Extraction and Biotinylation of IgG from Sera

P. tenuis organisms were digested in RIPA buffer and extracted protein was measured using BCA assay. Approximately 7 organisms were digested to obtain enough material for sufficient protein from the extraction.

To identify which of the proteins are interacting with IgG of infected animals, isolation of IgG from banked known positive samples was performed using a 1 mL NAb^{TM} Spin Kit. Three elutions were collected from this protocol. Buffer was

exchanged for PBS in each elution using a 100,000 MWCO Millipore ultrafiltration column for downstream applications. After purification and buffer exchange, a UV spectroscopy and a BCA assay were performed to assess concentration of antibodies in the elutions and determine the amount of biotin needed for successful biotinylation.

The antibodies were biotinylated using the protocol from the EZ-Link Sulfo-NHS-LC-Biotin kit with 20 molar excess of biotin to antibody. Excess biotin was removed using a 10,000 MWCO column using a pool of all three elutions. The concentrations were analyzed using spectroscopy to ensure appropriate levels have been maintained for downstream applications.

To assess if biotinylation was successful, ELISA on an avidin plate was performed (See Figure 3.2). Biotinylated antibody was used to coat 24 wells and another 24 wells were treated with PBS to serve as a blank. The plate was incubated at 37° C for 1 hour, washed, and then half of the PBS wells and half of the biotinylated antibody wells were treated with a secondary antibody which was chicken anti-cervid antibodies conjugated with horseradish peroxidase. The cells were incubated again for 1 hour at 37° C. After the plates were washed they were treated with TMB substrate, allowed to incubate at room temperature for 30 minutes and then the reaction was stopped using 0.18 molar sulfuric acid. The plates were read at 450 nm.

About 1.84 mg of biotinylated antibodies were then conjugated with 0.18 mg of extracted proteins and then passed over an avidin column for purification. Elutions collected were sent to the University of Georgia for identification of bound proteins using

liquid chromatography and mass spectrometry at their Proteomics and Mass Spectrometry Core Facility.

CHAPTER FOUR

RESULTS AND DISCUSSION

Production of the *Pt*-API-1 Protein

After cloning, the vector plasmid containing a *Pt*-API-1 insert was purified using the GeneJET Plasmid Miniprep Kit and yielded elevated levels of plasmid which could then be used to transform BL21 (DE3) *E. coli*.

Only the first elution during polyhistidine-tagged purification was ever suitable for use in downstream assays. Protein was confirmed with Western blot using anti-His secondary antibody and electrochemiluminescence (ECL) to visualize the bands.

Western Blot Diagnosis

The Western Blot Diagnosis of sera from animals with known infection status gave us a sensitivity of 0.96 and specificity of 0.78. This was significant enough for us to go on with tentative diagnostics of unknown sera if used in conjunction with clinical signs or positive necropsy.

However, the nature of the Western Blot itself, often left diagnosis in a gray area. It was easy to misinterpret a faint line as a negative and human nature tended to err on the side of diagnosing false positives. This was compounded by the issue of some known negatives testing as strong positives which was thought to be due to cross reactivity of similar parasites.

This prompted further investigation using the 10-mer synthetic overlapping peptides to identify regions of the antigen that were reacting with negative sera antibodies.

Testing with Synthetic Overlapping Peptides

Results obtained from the ELISAs with whole antigen overlapping peptide revealed several epitopes when probed with positive sera (See Figure 4.1). When compared with negative sera, however, it was evident that many of these epitopes were also reactive with antibodies from animals known not to be infected (See Figure 4.2). It was determined that the epitopes found in peptides 5-7 were promising enough to warrant designing more overlapping peptides focusing in that region. This would allow us to obtain higher quality peptides as well, hopefully solving some of the repeatability issues that had arose.

Results obtained from the ELISAs with the overlapping peptides at the previously identified epitope suggest reactive regions around peptides 15-21. Overall, the results suggest that there is significant cross-reactivity with similar parasites such as *O. ostertagi*, *D. viviparus*, and *H. contortus*. This is seen by the negative sample, N14-447 consistently reacting with these 3 peptides as well as having high reactivity with the *P. tenuis* antigen at peptides 4, 5, and 6 (see Figure 4.3).

The pretreatment of negative sera (N14- 423) and positive sera (PT+ 1 and PT+ 13) with peptides 22, 23, and 24 was not effective at eliminating the incidence of false positives. As seen in Figure 4.2, there was not a significant drop in the negative sample's reactivity after pretreatment and it still was showing consistently high results.

Furthermore, the positive samples also seemed to react with the antigen treatment, resulting in an unwanted decrease of their reactivity. This supports our theory of similar parasites inducing false positives in animals lacking any history of *P. tenuis* infection.

A dot blot confirmed the results obtained by the ELISAs. Peptides 5, 12, 13, 14, 15, 17, 22, and 24 were all reactive to banked positive sera (see Figure 4.5). Unfortunately, the reactivity with peptides 22 and 24 further emphasize the degree of cross reactivity with similar parasites.

Genomic Analysis for Investigation of Novel Antigens

DNA analysis showed that much of the sequenced data was deer DNA. The DNA was high quality, but with short reads and not many open reading frames. However, there were some sequences found that were identified as parasitic nematode antigens.

The RNA analysis proved to be much better with over 1000 transcripts and numerous open reading frames. It was also possible to perform in silico subtraction of the deer genome, making it much cleaner than the DNA results. Our BLAST results have already shown numerous unique proteins and 70 of the 1118 had no result at all in the database. As expected, many of these proposed proteins are hypothetical (not annotated) as genomes from this and similar parasites have not been reported. Top matches for the entire genome included parasites such as *D. viviparus, H. contortus,* and *Ancylostoma spp.* (See Figure 4.6).

The previously investigated aspartyl protease inhibitor was also sequenced in this transcriptome. BLAST results showed similarities of other nematode aspartyl protease inhibitors such as *Angiostrongylus cantnensis* (89.4%), *Dictyocaulus vivparus* (85.8%),

Trichostrongylus colubriformis (79.5%), *Ancylostoma duodenale* (84.0%), *Haemonchus contortus* (84.5 %), *Necator americanus* (87.8%), and *Ostertagia ostertagi* (86.7%).

The incomplete nature of all accessible databases makes significant identification of antigens difficult. BlastKOALA was arguably the most appropriate tool to obtain an overview of the functionality of the proteins discovered as it is used to annotate high-quality genomes. Of the 892 entries submitted, 518 were annotated (58.2%). Genetic information processing comprised around 45.69% of the entries annotated. Carbohydrate metabolism, energy metabolism, and environmental information processing comprised 8.1%, 7.9%, and 6.8% respectively (See Figure 4.7a). A GhostKOALA search was also performed despite our transcriptome being higher quality than a metagenome usually used in these searches. This was done because it was known that databases for nematodes are incomplete and GhostKOALA obtained a taxonomic breakdown of the data that could indicate the percentage of annotations that were correctly attributed to nematodes. The GhostKOALA came back with 540 (60.5%) annotations, roughly given the same functional category distribution as the more precise BlastKOALA search. Interestingly, 734 of the 892 sequences (82.3%) were correctly identified as nematodes. This left 149 (16.7%) undefined. The remaining <2% were Vertebrates, arthropods, eudicots, and basidiomycetes (See Figure 4.7b). It is assumed that many of those misidentified and undefined are the result of the incomplete nematode databases available or due to poor quality transcripts.

P. tenuis Protein Extraction and Biotinylation of IgG from Sera

An estimated 0.339 mg of protein was extracted from *P. tenuis* organisms when analyzed by BCA assay. Antibody elutions E1, E2, and E3 measured at 280 nm were 4.5, 4.60, and 3.01 mg respectively but after buffer exchange were significantly reduced to 1.3, 0.9, and 0.2 mg. This loss has been seen before using these columns and while frustrating, there was still plenty to use for downstream applications.

After biotinylation, a BCA assay using bovine serum albumin (BSA) as a standard revealed E1, E2, and E3 to be 1.73, 1.19, and 0.08 mg. The spectrometer read E1 as 3.35 mg, so it was assumed that the real concentration fell somewhere between 1.73 and 3.35 mg. The BSA standard likely does not react identically with the BCA assay as the protein of interest. Factors such as hydrophobicity and amino acid composition can influence color reaction outside of protein concentration (Wiechelman et al., 1988). Spectrometry, on the other hand, measures the concentration by detecting aromatic residues and does not require comparison to a standard curve.

Excess biotin was removed using a smaller 10,000 MWCO column in an attempt to reduce loss. Final concentrations for E1, E2, and E3 after removal of excess biotin in PBS measured 1.28, 0.56, and 0.11 mg respectively.

After biotinylation was confirmed, E1 and E2 were combined to achieve approximately 1.84 mg of antibody which was then incubated with 0.18 mg of the acquired protein at 37° C for 1 hour. Excess antibody to extracted *P. tenuis* protein should help with maximum binding of all possible antigens. After passing through the avidin column, the best elutions were found to be B2 and B3 (1.0 and 0.4 mg

respectively). These samples were sent to the University of Georgia for identification of bound proteins using liquid chromatography and mass spectrometry at their Proteomics and Mass Spectrometry Core Facility.

At present, results are pending, but several proteins have been identified from the data. An SCP-like extracellular protein has shown significant promise and shares minimal homology with similar nematodes. Its closest identity was 45% with *Ancylostoma ceylancium*, a type of hookworm. A synthetic gene for this protein has been created and efforts are underway to investigate its efficacy in diagnostic testing.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

Inability to Quantitively Diagnose Populations

The ability to assess prevalence, even in white-tailed deer populations, leaves much to be desired. The possibility of unisex infections increases the likelihood of obtaining false negatives during fecal exams (Slomke, Lankester et al. 1995). Additionally, the skill required during necropsy also will leave some misdiagnosed. If the person performing the necropsy fails to thoroughly open the venus sinuses along the meninges, many worms can go unnoticed.

This leaves us with many issues. Primarily, we are unable to accurately predict rate of infection or even infection status accurately. This information would be invaluable while creating an antemortem serological assay as we could obtain sera from a random selection of white-tailed deer and compare our observed prevalence in the sera to the known prevalence of the given area with a chi square test. With things how they are, however, this is impossible to do with any amount of accuracy.

Once a reliable serological diagnostic is acquired, we will have the ability to test collections of banked sera and perform retrospective studies on *P. tenuis* prevalence over time. This could provide insight on the rate that the parasite has spread across populations that presently impossible to assess.

Experimental Infections

Model organisms have been used in the past to aid with *P. tenuis* serological diagnosis. Immunized rats have been proven to produce antibodies against *P. tenuis* that can be detected and reliably diagnosed (Duffy, MacAfee et al. 2002).

Incorporation of model organisms in future research after another novel antigen would be useful. Mice and rats would be the logical starting point for such investigation, but further experimentation could even incorporate guinea pigs which have been shown to become naturally infected (Southard, Bender et al. 2013).

Cross Reactivity is a Significant and Underdiscussed Issue

Cross reactivity was a significant issue during this study and something that has been previously underdiscussed. *O. ostertagi* was previously found to have 74% sequence identity with the *Pt*-API-1 gene, however, in that same study, it was not determined whether exposure to *O. ostertagi* would produce a false positive (Duffy, MacAfee et al. 2002). From our own BLAST results, *O. ostertagi*, had 86.7% similarity with *P. tenuis* which could easily explain the cross reactivity.

When positive sera were pre-treated with *O. ostertagi*, *D. viviparus*, and *H. contortus* antigen, there was an overall decrease in binding to *P. tenuis* antigen, suggesting that the antibodies created against *P. tenuis* also had significant interactions with the aspartyl-protease inhibitors of these similar parasites (Fig. 4.2).

Further work needs to be done to identify an antigen that does not possess this degree of cross-reactivity. So far, we have compiled a transcriptome for *P. tenuis* and

have used banked positive sera to capture potential antigen targets extracted from whole L3 adult worms. Once analysis of these antigens is completed we will be able to cross reference them to our transcriptome and identify a gene for insertion into a plasmid vector and protein expression in competent *E. coli*.

Promising Results Obtained with *Elaeophora* Immunogenic Antigens

Using the same antigen capture protocol we used with *P. tenuis, Elaeophora schneideri* immunogenic antigens have been identified (see Figure 5.1) and are presently undergoing testing with known positive and negative sera. As with *P. tenuis*, many of the peptides identified yielded hypothetical proteins in the BLAST search due to the lack of genomic information in the database.

A source protein for a few of the peptides was identified from this data. Using specially designed primers, a PCR was performed to amplify the gene responsible for this source protein and was inserted into a plasmid vector for protein expression in *E. coli*.

Work with this organism is also presently ongoing, but the results have indicated similar techniques with *P. tenuis* should yield comparable results. Results from UGA have identified at least one promising new target that can be cross-identified to transcriptomic data. Presently, efforts to amplify this gene from purified *P. tenuis* DNA and insertion into a plasmid vector for protein expression is ongoing. Previous issues with insertion of an *Elaeophora* gene has led to the decision to construct a synthetic gene with codon optimization for expression in *E. coli*.

Competitive ELISAs

Ultimately, the diagnostic assay used to diagnose *P. tenuis* would employ a competitive ELISA that is compatible with a multitude of species. This way, the test used to diagnose alpacas would be identical to the one used to diagnose cervid species. This is particularly important as *P. tenuis* infects a wide variety of aberrant hosts including horses, goats, alpacas, moose, elk, and even guinea pigs (Southard, Bender et al. 2013).

Commercial production of this diagnostic assay would be valuable not only to wildlife veterinarians and biologists studying *P. tenuis* in cervid populations, but also a definitive diagnostic for farm animal and companion veterinarians in the clinical setting. Its applications could be useful for both monitoring and treating incidences of parelaphostrongylosis.

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APPENDIX

Table 4.1: Testing of sera with known *P. tenuis* status using western blot (WB). These results revealed a sensitivity of 0.96 and specificity of 0.78.

Sample ID	Expected Result	WB Result
N14-415	Negative	Negative
N14-419	Negative	Negative
N14-423	Negative	Positive
N14-425	Negative	Negative
N14-438	Negative	Negative
N14-447	Negative	Positive
N14-450	Negative	Negative
N14-455	Negative	Negative
N14-462	Negative	Negative
N14-466	Negative	Negative
N14-467	Negative	Negative
N14-468	Negative	Positive
N14-475	Negative	Negative
N14-476	Negative	Negative
PT+1	Positive	Positive
PT+2	Positive	Positive
PT+3	Positive	Positive
PT+4	Positive	Positive
PT+5	Positive	Positive
PT+6	Positive	Positive
PT+7	Positive	Positive
PT+8	Positive	Positive
PT+9	Positive	Positive

Table 4.1 continued

Sample ID	Expected Result	WB Result
PT+10	Positive	Positive
PT+11	Positive	Positive
PT+12	Positive	Positive
PT+13	Positive	Positive
PT+14	Positive	Positive
PT+15	Positive	Positive
PT+16	Positive	Positive
PT+17	Positive	Positive
PT+18	Positive	Positive
PT+19	Positive	Positive
PT+20	Positive	Positive
PT+21	Positive	Negative
PT+22	Positive	Positive
PT+23	Positive	Positive
PT+24	Positive	Positive
PT+25	Positive	Positive

1	2	3	4	5	6	7	8
9	10	11	12	13	14	15	16
17	18	19	20	21	22	23	24
PT+1 undiluted	PT+1 1:100	PT+1 1:200	PT+1 1:300	PT+1 1:400	PT+1 1:500	PT+1 1:600	PT+1 1:700
PT+3 undiluted	PT+3 1:100	PT+3 1:200	PT+3 1:300	PT+3 1:400	PT+3 1:500	PT+3 1:600	PT+3 1:700
PT+5 undiluted	PT+5 1:100	PT+5 1:200	PT+5 1:300	PT+5 1:400	PT+5 1:500	PT+5 1:600	PT+5 1:700

Figure 3.1: Schematic of dot blot used to assess the sera antibody reactivity to the *Pt*-API-1 overlapping synthetic peptides. Upper three rows were directly blotted with peptide indicated to assess primary antibody binding to antigen. Bottom three rows were directly blotted with sera indicated to ensure secondary antibody binding to primary antibody.

	PBS						Biotinylated Antibody					
PBS							E1	E1	E2	E2	E3	E3
							E1	E1	E2	E2	E3	E3
2°							E1	E1	E2	E2	E3	E3
							E1	E1	E2	E2	E3	E3

Figure 3.2: Schematic of ELISA done to assess biotinylation. None of the wells on the left half of the plate contained primary antibody (biotinylated antibody). Upper left-hand corner wells were PBS blanks with lower left-hand serving as a secondary antibody control. All wells on the right half of the plate contained primary antibody (elution of biotinylated antibody as depicted) so that each iteration had quadruplicates. Primary and secondary antibody together were only in the lower right-hand corner of the plate.

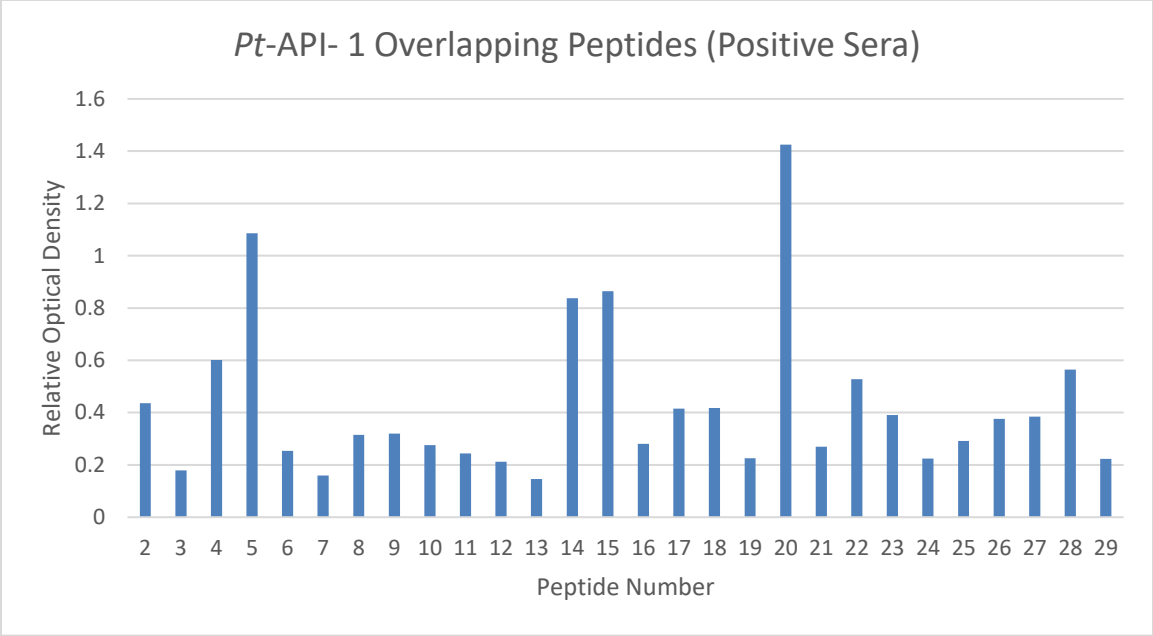


Figure 4.1: Epitopes identified using synthetic overlapping peptides of the whole antigen and known positive sera. Results indicate peaks around peptides 5, 14, 15, and 20. These suggest potential epitopes on the protein.

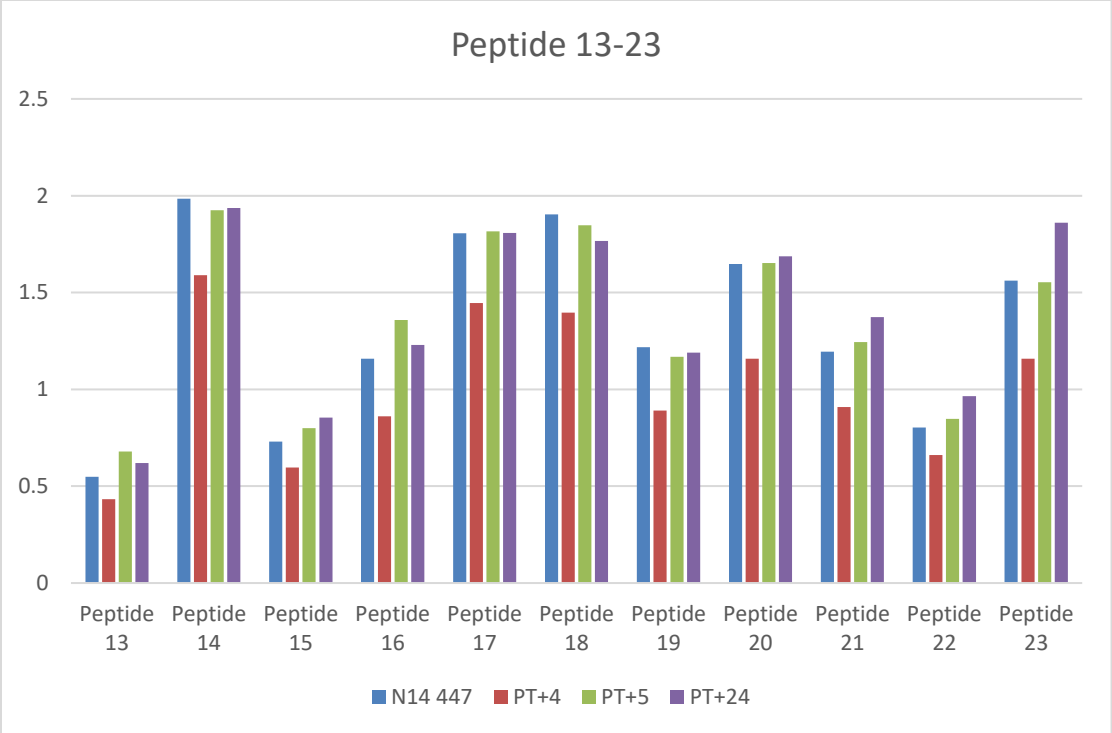
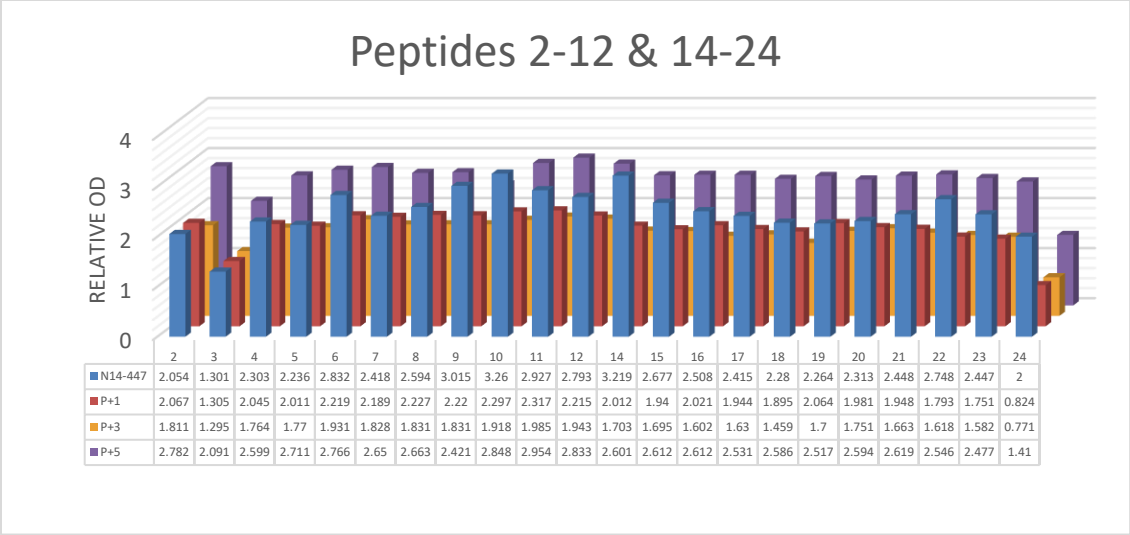


Figure 4.2: Comparison of negative sera reactivity (N14-447) and positive sera (PT+4, PT+5, PT+24) shows significant reactivity despite infection status being negative. Particularly, peptides 14 and 18 feature negative sera reacting stronger than all positive samples.



	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	18	19	20	21	22	23	24
N14-447	2.054	1.301	2.303	2.236	2.832	2.418	2.594	3.015	3.26	2.927	2.793	3.219	2.677	2.508	2.415	2.28	2.264	2.313	2.448	2.748	2.447	2
P+1	2.067	1.305	2.045	2.011	2.219	2.189	2.227	2.22	2.297	2.317	2.215	2.012	1.94	2.021	1.944	1.895	2.064	1.981	1.948	1.793	1.751	0.824
P+3	1.811	1.295	1.764	1.77	1.931	1.828	1.831	1.831	1.918	1.985	1.943	1.703	1.695	1.602	1.63	1.459	1.7	1.751	1.663	1.618	1.582	0.771
P+5	2.782	2.091	2.599	2.711	2.766	2.65	2.663	2.421	2.848	2.954	2.833	2.601	2.612	2.612	2.531	2.586	2.517	2.594	2.619	2.546	2.477	1.41

Figure 4.3: Testing sera reactivity across overlapping *Pt*-API-1peptides (2-21) and peptides of similar antigenically similar parasites (22-24). Peptide 22 is *O. ostertagia*, peptide 23 is *D. viviparus*, and peptide 24 is *H. contortus*. These result an area of interest around peptides 15-21, but also show significant cross-reactivity with antigens of similar parasites.

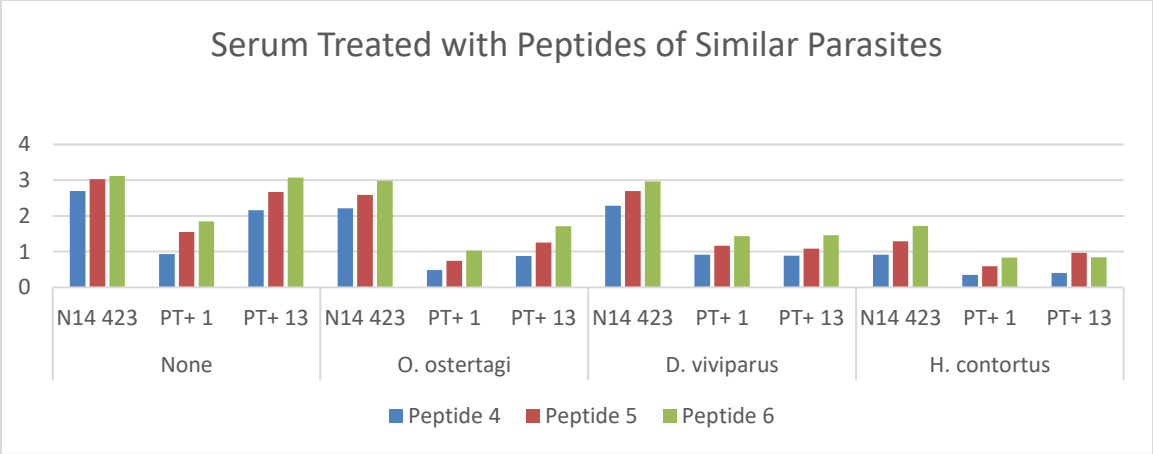


Figure 4.4: Testing sera pre-treated with peptides of similar parasites to attempt and reduce cross reactivity. Results show that regardless of pretreatment, cross reactive antibodies are not adequately absorbed by similar parasite peptides to reduce incidences of false positives.

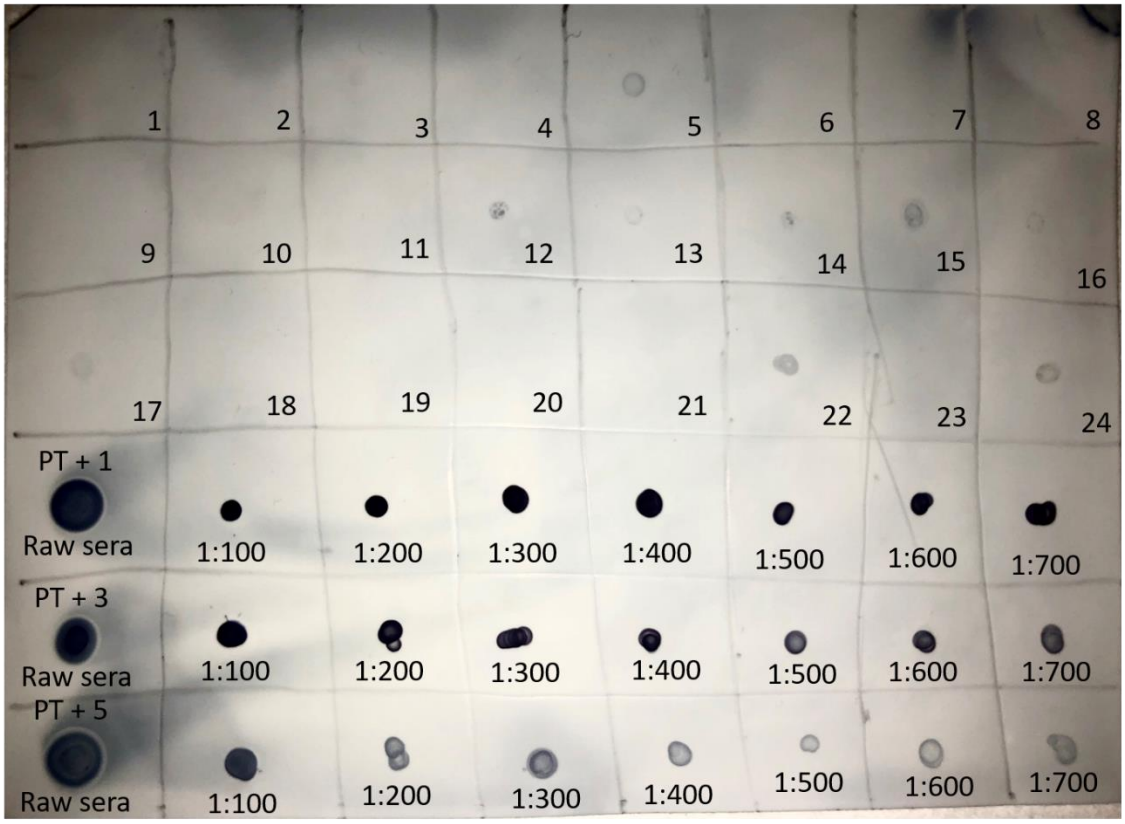


Figure 4.5: Dot blot performed with peptides 1-24 to assess positive sera binding to peptides. Peptides 3, 5, 12, 13, 14, 15, 16, 17, 22, and 24 all reacted with the pooled positive sera. The bottom three rows were blotted directly with serial dilutions of three selected sera to ensure the secondary antibody would bind to the serum antibodies.

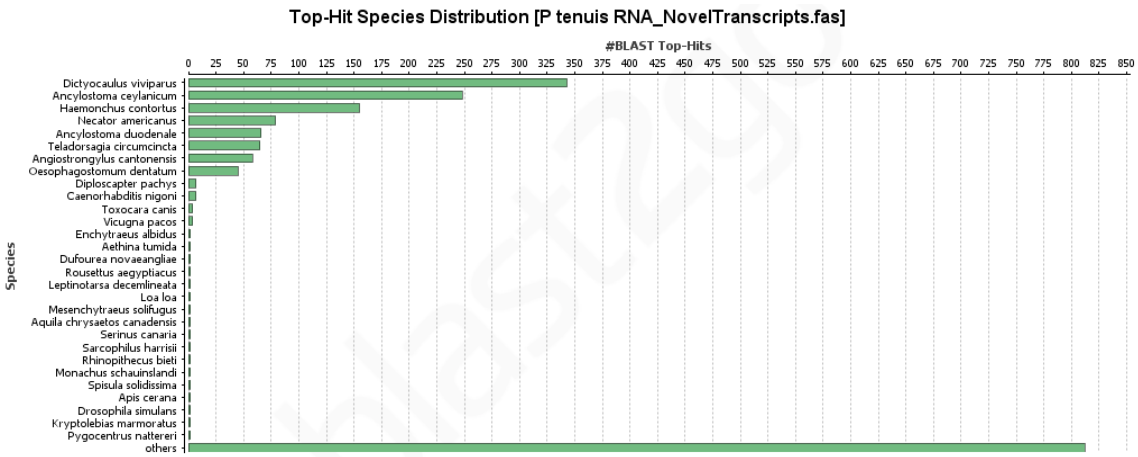


Figure 4.6: Top BLAST hits feature parasites seen to have cross reactivity with our antigen such as *D. viviparus* and *Haemonchus contortus*. Absence of *O. ostertagia* in this constructed graph is probably due to an insufficient database.

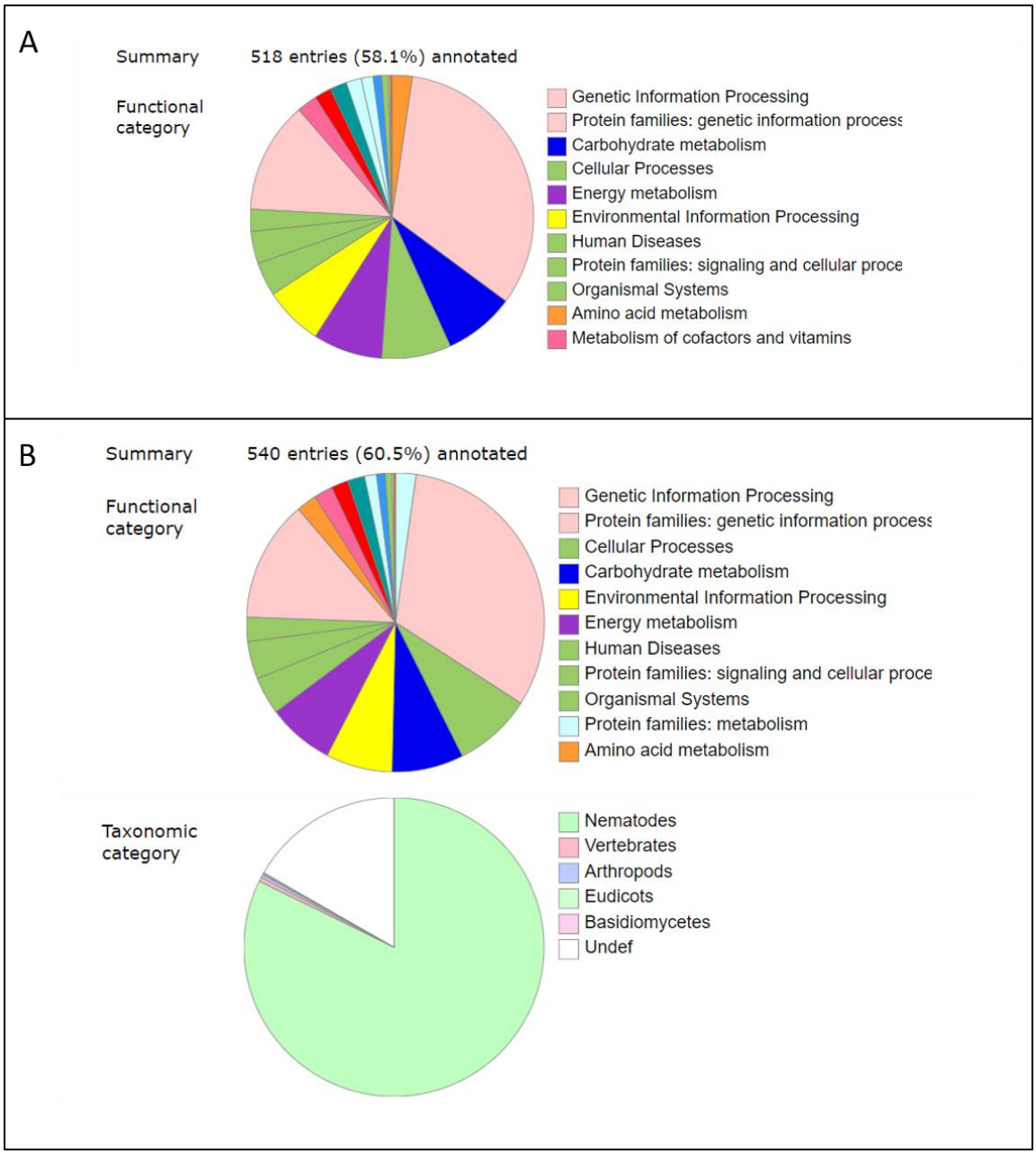


Figure 4.7: KEGG Orthology and Links Annotation results. BlastKOALA (A) returned 518 entries (58.1%) annotated into functional categoreis. GhostKOALA returned 540 entires (60.5%) annotated into functional categories with the majority falling under the nematode taxxonomic category.

Peptide Sequence	Closest BLAST Match	E Value	Max Score	Query Cover
VSTQSTGIQESQRN CPKMSTRTTAGG	Predicted: Low Quality Protein: 26S proteasome non-ATPase regulatory subunit 7	8.6	34.1	42%
SQRNCPKMSTRTT AGG	Hypothetical protein, variant [Loa loa]	46	30.3	56%
HTESHEVTTPQGQ TSDSAEAH	OmdA domain-containing protein (Streptomyces europaeiscabei)	0.14	38.4	75%
VQQQTPQQQQQQ QKKGPTVPAKPGQ TPITR	Hypothetical protein X798-00587 (Onchocerca flexuosa)	4e-11	67.2	85%
TESHEVTTPQGQT SDSAEAH	OmdA domain-containing protein (Streptomyces europaeiscabiei)	0.12	38.4	75%

Figure 5.1: Immunogenic peptides found in *Elaeophora schneideri* after antigen capture with positive sera.

VITA

Jessie Erin Richards was born May 6, 1994 in Nashville, Tennessee, and grew up in the small town of Cross Plains, Tennessee. She attended high school at East Robertson High School in Cross Plains and graduated in 2012.

She was accepted to the University of Tennessee, Knoxville and majored in Animal Science with a concentration in Pre-veterinary Medicine and minor in Biological Sciences. During this time, she participated in the Comparative & Experimental Medicine's undergraduate research program and was fortunate enough to work with Dr. Stephen Kania with the creation of a serological assay for *Parelaphostrongylus tenuis.* This piqued her interest in Immunology and further classes with Dr. Sarah Lebeis solidified her decision to pursue it as a career.

Jessie received her Bachelor of Science May 2016. Unfortunately, her first attempt to apply for veterinary school was not successful and, thus, entered the Comparative & Experimental Medicine program at UT as a Masters student per the encouragement of her mentor, Dr. Kania. Her chosen field of study was parasite immunology and she chose Dr. Richard Gerhold as her primary advisor with Dr. Kania and Dr. Lebeis also serving on her committee.

The following year, she was accepted into UT's College of Veterinary Medicine class of 2021 and began her studies as a dual DVM/Masters student. During her second semester as a DVM student, she was accepted into the dual DVM/PhD program and it was determined that her Masters coursework would contribute towards a concurrent PhD.

Jessie's research, since her undergraduate experience, has focused on the creation of a serological diagnostic assay for *P. tenuis*. It is her hope that someday her research will contribute to the creation of a practical antemortem test in a variety of species. The work in this thesis further investigates antigens that would prove appropriate for such an assay and reinforces the need for the identification of an antigen with limited cross-reactivity with similar strongyle species.