INVESTIGATION OF A METHOD TO REDUCE FALSE-POSITIVE EQUINE PROTOZOAL MYELOENCEPHALITIS TEST RESULTS

By

KATHERINE ANNE HESKETT

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2004

This document is dedicated to my husband, Eric, for the never ending support and guidance he gave me while completing this work; to my entire family, especially Mom, Dad, Shelia, Eddie, and Ronnie, for their support, love, and belief in me; and to Judy for all her love and support, she will always be with me.

ACKNOWLEDGMENTS

First I would like to express my sincere appreciation to my supervisory committee chairman, Dr. Robert MacKay, for his support and guidance throughout this project. I am grateful for all Dr. MacKay has done for me, has taught me, and support he has given to me throughout my graduate education.

I would also like to express my sincere thanks to my committee members, Dr. Maureen Long and Dr. David Allred, for all their support. They were an endless resource for me while completing this project. Their time and patience with me while completing this project were tremendous.

I also extend a sincere thank you to Tim Massey, Dwight Schroedter, Brent Mayer, John Malley, and Chris Stivers from Neogen Corporation for performing the RID assays on our project samples.

Karen Gillis deserves a special thank you for all the patience and support she provided to me during this project. Her technical assistance and background knowledge was essential to me in laboratory. A thank you also goes to Linda Lee-Ambrose for her assistance in the laboratory as well.

I must extend a thank you to Dr. Charles Courtney, Associate Dean for Research and Graduate Studies at the College of Veterinary Medicine, for his support of me through my studies as a graduate student. Also in the office for the dean I must thank Sally O'Connell for all her time and attention to detail needed to meet the degree requirements and deadlines.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure page

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

INVESTIGATION OF A METHOD TO REDUCE FALSE-POSITIVE EQUINE PROTOZOAL MYELOENCEPHALITIS TEST RESULTS

By

Katherine Anne Heskett

August 2004

Chair: Robert J. MacKay
Major Department: Veterinary Medicine

Equine protozoal myeloencephalitis (EPM) is a neurological disease of horses that has been recognized since the early 1960s. Clinical signs range from mild lameness to recumbency to sudden death. EPM is mainly caused by the protozoan *S. neurona*. It is suggested that at least 50% of the horses in the United States have been exposed to *S. neurona* while only a small amount of these horses will develop clinical disease. The decreased performance potential, treatment expenses, and possible loss of life cost the industry millions of dollars annually.

Current diagnosis depends on the detection of *Sarcocystis neurona* antibodies in the serum and cerebrospinal fluid (CSF) by immunoblot. Antibodies present in the CSF are believed to be indicative of active infection, whereas antibodies in the serum are believed to be indicative of exposure to *S. neurona*. Since the immunoblot can not distinguish between antibodies that are produced in the CNS versus those that come from the blood, false positive results are commonly obtained from CSF contaminated with blood.

The intent of this project is to explore a new method in the diagnosis of EPM. The main objectives of this study were to develop an enzyme linked immunosorbent assay (ELISA) for total equine IgG and for IgG antibodies against *S. neurona*, for both serum and CSF. Standard curves were established for each assay and IgG concentrations and *S. neurona* antibody titers for serum and CSF were determined. The concentrations from each assay were used together to measure a specific CSF coefficient (C) for detection of CNS produced anti-*S. neurona* IgG. These assays were used on experimental and clinical samples available. C values were calculated and analyzed for both sets of samples.

CHAPTER 1
INTRODUCTION

The equine industries are highly diverse and contribute generously to the United States economy. The industries range from breeding, training, maintaining, and riding horses to operating racetracks, horse shows, and public sales (American Horse Council). According to the American Horse Council, the equine industry is worth $112 billion and provides 1.4 million jobs. In 1995, exports of horse carcasses were estimated at $67.5 million and exports of live horses were estimated at $285 million (US Department of Agriculture (USDA), Animal Plant Health Inspection Service Report (APHIS), June 1996).

Equine protozoal myeloencephalitis (EPM), originally called segmental myelitis or focal myelitis-encephalitis, is a neurologic disease of horses that has been recognized in this country since the early 1960s (Rooney, 1969). This disease may be progressive and may include clinical signs ranging from mild lameness to recumbency or, in rare instances, death.

In 1997, the USDA APHIS report showed that EPM was ranked by owners as a number one concern for those infectious diseases listed (USDA, NAHMS Report May 1997), though it is now thought that West Nile Encephalomyelitis may be ranked above EPM. As of 2001, the cost just for diagnosis and treatment of EPM in the US ranged from $55.4 to $110.8 million per year (Dubey et al., 2001b).

Testing for EPM is currently performed by three commercial laboratories using an immunoblot (Granstrom et al., 1993) test for IgG against Sarcocystis neurona, the

organism that causes most cases of EPM (Dubey et al., 1991). Serum and CSF samples may be submitted for testing. The three laboratories are Equine Biodiagnostics Incorporated (EBI, Lexington, KY), Neogen Corporation (Lexington, KY), and the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI). There has also been a study recently described using a serum indirect fluorescent antibody test (IFAT) at the University of California Davis (Duarte et al., 2003). The immunoblot test has good sensitivity but poor specificity (many false-positives); thus, its principal value is in identifying those horses that are truly negative. The IFAT test yielded 100% specificity for diagnosis of EPM (Duarte et al., 2003), however, this test was only performed on serum, and serum is used to indicate exposure only.

Cserr and Knopf (1992) contend that there normally is traffic of IgG from blood into CSF in the absence of CNS disease. Therefore, in horses without CNS infection by *S. neurona*, it is hypothesized that *S. neurona* IgG is found in CSF in the same proportion to total IgG as is the case in blood in the same horse. According to this hypothesis, the ratio of *S. neurona*-reactive IgG/total IgG in CSF will only exceed that in blood when there is a true CNS infection by *S. neurona*. It is this hypothesis that provided the basis of our study. We aimed to distinguish between anti-*S. neurona* IgG which is produced in the CNS and that which is produced systemically. To do this, we explored the use of an enzyme linked immunosorbent assay (ELISA) to quantify total IgG and *S. neurona*-reactive IgG. We then used the values obtained from these ELISAs to create a specific CSF coefficient (C, also known as Goldmann-Witmer coefficient). This coefficient has been used previously to distinguish antibodies in aqueous humor produced locally (i.e., in

ocular tissues) from those produced systemically (Lappin et al., 1996b). The coefficient compares the proportions of specific (i.e., anti-*S. neurona*) antibody in a tissue space (e.g., CSF, joint, peritoneal cavity) and serum.

EPM is still an important neurologic disease in this country. Its effects can impact the industry economically and cause a major emotional toll on owners. We hope through our objective of developing a more specific diagnostic test via the use of the specific CSF coefficient that we can make a difference in the area of EPM diagnosis.

CHAPTER 2
LITERATURE REVIEW

Equine Protozoal Myeloencephalitis (EPM)

Equine protozoal myeloencephalitis (EPM) is a neurological disease of horses in the Americas. In 2001, Dubey and colleagues (Dubey et al., 2001b) reported that EPM was ranked as the most commonly diagnosed infectious neurological disease of horses in America.

History

A new neurological syndrome was first identified in the 1960s by Rooney et al. (1969). It was described as segmental myelitis or focal myelitis-encephalitis (Rooney et al., 1969). In 1974, Cusick and colleagues (Cusick et al., 1974) described the histopathology of the disease and determined that protozoa were associated with the lesions. The authors identified these organisms as *Toxoplasma gondii* (Cusick et al., 1974). Two years later, however, it was suggested that the protozoa actually were a *Sarcocystis*-like organism and not *Toxoplasma* (Dubey, 1976). Using electron microscopy, Simpson and Mayhew (1980), supported this interpretation on the basis of the organism's ultrastructure. The name "equine protozoal myeloencephalitis" was given to the neurological condition in 1976 by Mayhew and colleagues (Mayhew et al., 1976). In 1991, Dubey and colleagues cultured a protozoan from the spinal cord of a naturally infected horse and named it *Sarcocystis neurona* (Dubey et al., 1991). Many additional isolates of *S. neurona*, from the spinal cords of horses with neurological signs, have been reported since 1991 (Davis et al., 1991a, 1991b; Dubey et al., 1999, 2001b, & c;

Granstrom et al., 1992, 1994; Liang et al., 1998, Mansfield et al., 2001, Marsh et al., 1996a, 1997, 2001).

Clinical Signs

Depending on where *Sarcocystis neurona* attacks (it has the ability to attack both the white and gray matter of the CNS) and the severity of damage it causes, varying clinical signs can be seen. Damage to the brain or brain stem may result in depression, head tilt, facial paralysis, and difficulty in swallowing, whereas damage to the spinal cord may result in gait abnormalities and ataxia (MacKay, et al., 2000). Focal muscle atrophy, severe muscle weakness, and loss of reflexes are some other common signs seen with EPM. EPM is often a progressive disease in which clinical signs may vary from acute to chronic, may stabilize, worsen suddenly, or relapse after a period of improvement (MacKay et al., 2000).

Demographics/Epidemiology

The distribution of EPM follows that of the definitive host for *S. neurona*, the opossum. The opossum is widespread in the United States. It has been estimated that approximately 50% of the horses in the United States have been exposed to *S. neurona*, on the basis of the presence of serum antibodies to *S. neurona* (MacKay et al., 2000). The prevalence of EPM, however, is estimated to be less than 1%, on the basis of postmortem findings (Granstrom, 1997, NAHMS, 1998) and on owner-reported diagnoses (NAHMS, 1998).

EPM affects primarily young horses. A study at the Ohio State University showed that young horses (1-5 years old) and older horses (>13 years old) were at an increased risk for developing EPM (Saville et al., 2000a). Additional risk factors for EPM were also found. An increased risk of developing EPM was seen with conditions that may

predispose to immune suppression, including non-neurologic disease, advanced age, and other forms of stress (Saville et al., 2000b). Possibly because of their high level of stress, racehorses and show horses are at a higher risk for developing EPM when compared to breeding and pleasure horses (Saville et al., 2000a; Saville et al., 2000b).

Saville and colleagues found that there was a higher risk of developing EPM among horses admitted to a veterinary teaching hospital during the spring, summer, and fall as compared with those admitted during the winter (Saville et al., 2000b). This may be due to close contact with opossums, the survival of *S. neurona* in the environment (Saville, et al., 1997), or the stress induced by the timing of competition events (MacKay, et al., 2000). In this study, other risk factors associated with an increase of EPM included observed presence of opossums, presence of wooded terrain around the farm, and previous diagnosis of EPM on the farm (Saville, et al., 2000b).

In the NAHMS Equine '98 study, EPM was shown to be more likely to occur on premises where opossums were often or sometimes identified (NAHMS 1998). The feeding of purchased grain compared to the feeding of home-grown grain was also associated with an increased risk of EPM in the NAHMS study.

Life Cycle of *Sarcocystis neurona*

In general, *Sarcocystis* species have a 2-host life cycle, alternating between definitive and intermediate hosts (Dubey, 1976). The definitive host (a carnivore) eats sarcocyst-infested tissue from an intermediate host (MacKay et al., 2000).

In 1995, Fenger and colleagues proposed that the definitive host for *S. neurona* was the Virginia opossum, *Didelphis virginiana*. Fenger and colleagues used polymerase chain reaction (PCR) to amplify the small subunit (18S) ribosomal RNA gene from sporocysts found in intestinal tracts of various possible definitive hosts, including

opossums, raccoons, skunks, cats, a red-tailed hawk, and a coyote. The gene sequence obtained from opossum sporocysts was highly similar (99.89%) to that of *S. neurona* (Fenger et al., 1995). Further evidence for the role of the opossum as definitive host was the observation by Fenger and colleagues (1997) that horses given opossum sporocysts produced *S. neurona* antibodies in blood and CSF, showed signs of neurologic disease, and had histopathologic lesions compatible with EPM (Fenger et al., 1997). However, organisms were not recovered from the CNS of these horses.

Consistent with the general life cycle schema for *Sarcocystis* species, the life cycle of *S. neurona* is advanced once the definitive host, the opossum, eats sarcocyst-infected muscle from an intermediate host. The intermediate host(s) was (were) not known for some time after the initial discovery of *S. neurona*. Sarcocysts were dissected from armadillo tongues and DNA was extracted and characterized by PCR amplification and restriction endonuclease digestion (Tanhauser et al., 2001). Amplified sequences were highly similar to those of *S. neurona*. In addition, an immunoblot technique to detect *S. neurona* antibody was developed and used on armadillo plasma. All wild-caught armadillos in this study contained antibodies to *S. neurona*. The results from this study suggest that the nine-banded armadillo (*Dasypus novemcinctus*) is a naturally-infected intermediate host for *S. neurona* (Tanhauser et al., 2001). Further support for this notion was the observation by Cheadle et al. (2001a) that opossums fed armadillo muscles in turn shed *S. neurona* sporocysts in their feces. Sporocysts were identified as *S. neurona* by PCR and DNA sequencing (Cheadle et al., 2001a). The striped skunk (*Mephitis mephitis*) was determined to be an experimental intermediate host for *S. neurona* (Cheadle et al., 2001b). Skunks that were inoculated with *S. neurona* sporocysts from a

naturally infected opossum formed sarcocysts in skeletal muscle (Cheadle et al., 2001b). In turn, skunk muscles containing sporocysts were fed to laboratory-reared opossums which then shed *S. neurona* sporocysts. The sarcocysts from the skunks and sporocysts from the opossums were identified as *S. neurona* by using PCR and DNA sequencing (Cheadle et al., 2001b). Using strategies and techniques similar to those already discussed, Dubey and colleagues (2001d) identified the raccoon (*Procyon lotor*) as a natural intermediate host. Lindsay and colleagues (2000) found sea otters (*Enhydra lutris*) naturally infected with *S. neurona* – this species apparently also can function as an intermediate host. *S. neurona* infections have also been reported in Pacific harbor seals (Lapointe et. al, 1998). The current list of intermediate hosts includes armadillos, skunks, raccoon, sea otters, and Pacific harbor seals.

It has been found that the domestic house cat, *Felis domesticus*, is an experimental intermediate host (Ohio State web site, Dubey et al, 2000). In the study performed by Dubey and colleagues (2000), laboratory raised domestic cats (from a parasite-free colony) were fed sporocysts from the intestine of a naturally infected opossum (*D. virginiana*). These cats, some of which were immunosuppressed by cortisone, developed sarcocysts in muscles which were determined to be *S. neurona* on the basis of antigenic, molecular, and biological findings (Dubey et al, 2000). In turn, these infected cat muscles were fed to laboratory-raised opossums. These opossums then shed sporocysts in the feces (Dubey et al., 2000). Interferon γ knockout mice were fed sporocysts from intestinal scrapings of these opossums and were found to have *S. neurona* parasites in their tissues. Infected brain tissue from one of the cats was inoculated in cultured bovine turbinate (BT) cells and merozoites and schizonts developed. Sporocysts from one of

these opossums were also administered to two ponies which then seroconverted against *S. neurona* as determined by immunoblot testing (Dubey et al, 2000). In 2001, a small pilot study was conducted in Missouri to evaluate the cat's role as an intermediate host for *S. neurona* (Turray et al., 2002). Only 1/9 feral cats had serum *S. neurona* antibodies on immunoblot. Muscle from this cat was fed to an opossum which shed sporocysts which, in turn, successfully infected interferon-γ knockout mice (Turray et al., 2002). On the basis of the results of this study, Turray and colleagues suggest that the domestic cat may serve as an intermediate host for *S. neurona* (Turray et al., 2002). A more extensive study was carried out in Florida in 2003 (Gillis et al., 2003). Free roaming cats were examined for the presence of muscle sarcocysts and serum antibodies to *S. neurona* (Gillis et al., 2003). Ten percent of the cats (5/50) were found to have sarcocysts and 5% were found to have *S. neurona* antibodies. It was determined by morphologic, molecular, and biological means that these sarcocysts were not *S. neurona* or *S. neurona*-like (Gillis et al., 2003). On the basis of morphology and DNA sequence analysis, the sarcocysts were determined to be *S. felis*; however, immunoblot results indicated that there was possible co-infection with *S. neurona* (Gillis et al., 2003). The study by Gillis et al. (2003) concluded that further work is needed to determine to role of the domestic cat in the life cycle of *S. neurona*.

The horse is considered to be an aberrant dead-end host for *S. neurona* although little effort has been made to find *S. neurona* sarcocysts in horses. The horse ingests sporocysts from an infected opossum via contaminated feed, hay or water sources.

Neospora Related EPM

Neospora was first reported in a horse that was clinically diagnosed with EPM by Marsh and colleagues in 1996 (Marsh et al., 1996b). There have been additional cases of

EPM caused by *Neospora* sp (Hamir et al., 1998; Cheadle et al., 1999; Daft et al., 1997). The organism reacts with antisera against *N. caninum* but due to significant differences in the amino acid structure of two immunodominant surface antigens (Marsh et al., 1999) and nucleotide sequence differences in the first internal transcribed spacer region of the rRNA gene (Marsh et al., 1998), the organism that infects horses was classified as a separate species and named *N. hughesi*.

Clinical Diagnosis

EPM is a possible differential diagnosis in any horse exhibiting signs of CNS disease (MacKay et al., 2000). A complete neurologic examination and appropriate laboratory tests should be performed to determine the exact diagnosis.

Distinguishing EPM from other neurologic diseases can be difficult On the basis of the presenting clinical signs. Cervical vertebral malformation, equine herpesvirus-1 myeloencephalopathy, equine motor neuron disease, extradural and spinal cord tumors, epidural abscesses, migrating metazoan parasites, rabies, West Nile viral encephalomyelitis, equine degenerative myeloencephalopathy, vascular malformations, and discospondylopathies are other diseases to consider when confronted with a horse with signs compatible with EPM (MacKay et al., 2000).

Blood and CSF Analysis

Consistent changes in complete and differential blood cell counts or serum chemistry values are not seen in cases of EPM (MacKay, 1997a). CSF analyses, cytologic and chemical, do not always show abnormalities with EPM either, although some cases have mononuclear pleocytosis and high protein concentration (MacKay et al., 2000). CSF analysis may be most helpful in excluding EPM by suggesting the presence of neurologic diseases other than EPM (MacKay et al., 2000).

Postmortem Diagnosis

Definitive diagnosis, or confirmation of diagnosis, of EPM can only be made on post mortem examination. At necropsy, gross lesions of the CNS may be seen in horses with EPM, with lesions more often seen in the spinal cord than in the brain (Dubey et al., 2001b). Protozoa may be observed in these lesions; however, they are not always seen. Protozoa were seen in only 10 – 36% of H&E stained and 20 – 51% of immunostained sections (Boy et al., 1990; Hamir et al., 1998). Typically, lesions of EPM are characterized as having multifocal to coalescing areas of hemorrhage, nonsuppurative inflammation, and small foci of necrosis. Perivascular cuffing by mononuclear cells, infiltrates of lymphocytes, neutrophils, eosinophils, multinucleate giant cells, gitter cell formation, and astrocyte proliferation are also characteristic of lesions associated with EPM (MacKay et al., 2000; Dubey et al., 2001b).

Polymerase Chain Reaction Assay

The polymerase chain reaction assay was developed in 1994 by Fenger and colleagues. It was developed to detect parasite-specific DNA in equine CSF. PCR detects *S. neurona*-specific DNA by the amplification of the 18S gene of the parasite. The sensitivity of this assay is not as great as originally projected (MacKay et al., 2000). This may be due to the lack of parasites in the CSF or to destruction of parasite DNA by enzymatic action of inflammatory CSF (Marsh et al., 1999).

Immunoblot Test

An immunoblot, also referred to as a Western blot, test is used to detect the presence of anti- *S. neurona* IgG in the serum or CSF of an infected horse. This test is used, therefore, as an aid in the diagnosis of EPM. It is accepted that a positive serum immunoblot suggests prior exposure to *S. neurona*. It is also accepted that a positive

immunoblot in a cleanly obtained CSF sample is suggestive of recent or active CNS exposure. Contamination of CSF by immunoblot-positive blood is considered to be a common and important cause of false-positive CSF immunoblot results. A positive CSF test result on a clean sample implies intrathecal production of *S. neurona* antibody in response to CNS infection and thus is supportive of but not definitive for the diagnosis of EPM (MacKay et al., 2000).

The immunoblot was developed by Granstrom and colleagues in the early 1990s (Granstrom et al., 1993). By using *S. neurona* merozoites and sera from horses with EPM (diagnosed histologically), a horse with an experimental *S. fayeri* infection, and rabbits inoculated with *S. neurona, S. cruzi,* and *S. muris*, a set of eight *S. neurona*-specific proteins were identified (Granstrom et al., 1993). A subset of these proteins, were used as the basis for the immunoblot test used commercially to diagnosis EPM.

Currently this test is commercially available for EPM testing at Equine Biodiagnostics Incorporated (EBI, Lexington, KY), Neogen Corporation (Lexington, KY), and the Diagnostic Center for Population and Animal Health at Michigan State University (MSU) (East Lansing, MI). Results of the immunoblot are reported as positive, non-specific, or negative. Positive results are those reported for samples that react with *S. neurona* proteins of certain specificity and negative results are for those that have no *S. neurona*-specific antibody detected. The non-specific results are generally categorized as negative results. The three laboratories that perform immunoblots for *S. neurona* IgG have different interpretations of the results depending on the proteins detected. At Neogen, a reaction against a 17-kDa band is considered positive while reaction against a 30-kDa band is considered a non-specific result. In addition to

immunoblot results, Neogen also semi-quantifies IgG in CSF against the specific 17-kDa *S. neurona* antigen. Dilutions of a standard positive equine serum are used to create a plot of band density vs. dilution (represented by a unitless scale of 0-100). Relative anti-17-kDa concentrations in test samples (called Relative Quantity CSF; RQ) are then estimated by interpolation into the standard curve. At EBI, reactivity against the protein bands of 14.5, 13, and 7-kDa indicate a positive result. At MSU reactivity against protein bands of both 30 and 16-kDa indicate a positive result.

Granstrom (1997) described a retrospective postmortem study of 295 horses euthanized for neurologic disease. The sensitivity of the test, which is the ability of the test to detect true positives, or horses that are diseased and test positive for EPM, was 89%; hence an 11% false-negative rate. The specificity, which is the ability of the test to detect true negatives, or horses that are not diseased and test negative for EPM, was also 89%, hence an 11% false positive rate. The positive predictive value (PPV), defined as the proportion of horses testing positive that are truly diseased in that population, was 85%. The negative predictive value (NPV), defined as the proportion of horses testing negative that are truly free of disease, in that population was 92%.

In 2000, Rossano and colleagues reported the development of a modified immunoblot test for antibodies to *S. neurona*. In an attempt to increase specificity, blots are incubated with sera from cattle which are naturally exposed to *S. cruzi* prior to sample addition. Samples are considered positive if they react with bands at both 30- and 16-kDa. These modifications were reported to increase specificity and sensitivity to almost 100%; however, this concept has been criticized on various grounds (Dubey et al., 2001b)

including the fact that *S. cruzi* does not infect the horse and the horse is not known to produce antibodies to *S. cruzi* (Dubey et al., 1989).

Daft and colleagues (2002) evaluated immunoblots of both CSF and serum from horses exhibiting neurologic signs and from clinically normal horses. Immunoblot results were compared to the post mortem immunohistochemistry results. In this study, 65 horses with neurologic signs and 169 horses without neurologic signs (clinically normal) were examined. The sensitivity of the serum and CSF immunoblots for detection of histologically diagnosed EPM was high, ranging from 80-88% in both neurologic horses and clinically normal horses. The specificity, however, was poor. The specificity in CSF of neurologic horses was 44%, while the specificity in CSF of normal horses was 60%. In serum the specificity was also low; 38% for neurologic horses and 56% for normal horses.

The high sensitivity in the study by Daft and colleagues yields a low false-negative rate for the diagnosis of EPM. Therefore, the test would be most useful in ruling out EPM. However, the low specificity of the test actually yields many false-positive results. These false-positives were decreased when weak reactive samples were interpreted as negative (Daft et al., 2002). Daft and colleagues suggest the lower specificity of the test in neurologic horses may be due to increased blood-brain barrier permeability due to another disease other than EPM. It was concluded from this study that the immunoblot is only useful for excluding the diagnosis of EPM. A possible criticism of this study is that the "gold standard" method of diagnosis used – namely, immunohistochemistry – may underestimate the number of cases of EPM, thus falsely lowering the specificity values obtained.

MacKay (1997a) describes some possible reasons for the false-positives on a CSF immunoblot test for EPM. Serologic studies have suggested that approximately 50% of the horses in the United States have been exposed to *S. neurona* and are serum positive (MacKay, 1997b). Therefore a CSF sample that is contaminated with blood during collection may yield a false-positive result. There is normal traffic of proteins from blood into CSF through the blood-brain barrier (Cserr et al., 1992). When this barrier is compromised due to a neurologic condition or disease (such as equid herpesvirus-1 myeloencephalopathy or trauma) there may be an increase in permeability of the barrier and therefore an increase of serum proteins in the CSF. Hence, this may also yield a false-positive EPM test result. There may also be a question of cross reactive antibodies being produced in the CSF. Another general cause for a false-positive EPM test result may be simply due to laboratory or technical error with the submitted sample.

Indirect Fluorescent Antibody Test

In 2003, Duarte and colleagues compared the results of an indirect fluorescent antibody test (IFAT) with those from the immunoblot and the modified immunoblot in serum from horses. The IFAT uses merozoites of the UCD-1 *S. neurona* isolate as the test antigen. Once the antigen is fixed to the wells of a slide, fluorescein-labeled affinity purified antibodies directed against horse-specific IgG are added, followed by the addition of serum samples (Duarte et al., 2003). On the basis of results obtained by immunohistochemical staining of CNS sections, horses were diagnosed as either being EPM-negative or EPM-positive. A total of 48 serum samples were selected for this study; 9 were from positive horses and 39 from negative horses (Duarte et al., 2003). The serum samples from the 48 horses were evaluated on each of the tests – IFAT, immunoblot (performed by Equine Biodiagnotics Inc.), and modified immunoblot

(performed by Diagnostic Center for Population and Animal Health at Michigan State University). All three tests yielded sensitivity for diagnosis of EPM of 88.9%. The immunoblot yielded a specificity of 87.2%, the modified immunoblot yielded a specificity of 69.2%, and the IFAT yielded a specificity of 100% (Duarte et al., 2003).

Duarte and colleagues performed a study which evaluated the IFAT on both serum and CSF samples of horses with EPM or experimentally infected with *S. neurona* (Duarte et al., 2004). The IFAT yielded a sensitivity of 83.3% for serum and 100% for CSF and a specificity of 96.9% for serum and 99% for CSF (Duarte et al., 2004).

Treatment

Horses that receive treatment for EPM are 10 times more likely to improve and those that do improve are 50 times more likely to survive (Saville et al., 2000a). If treatment is started as quickly as possible after clinical signs are recognized, treatment may be up to 75% effective (Dubey et al., 2001b). The standard traditional treatment has been the use of folate-inhibiting drugs such as sulfadiazine and pyrimethamine combinations. Horses are often treated for at least six months with this combination treatment. Side effects from treatment may include anemia, leukopenia, or neutropenia (MacKay et al., 2000).

Anti-cocccidial triazine compounds have also been used as treatment for EPM. These compounds, including diclazuril, toltrazuril, and its sulfone metabolite, ponazuril are all relatively new therapies (MacKay et al., 2000). These compounds must be administered for weeks at a time and therefore may become costly for a course of treatment. Ponazuril (Marquis®, Bayer Animal Health) is FDA approved for the specific use of EPM treatment.

Another treatment for EPM is nitazoxanide (NTZ), which is a benzamide compound with broad spectrum activity against protozoa, nematodes, cestodes, trematodes, and bacteria (MacKay et al., 2000). In toxicity studies anorexia, depression, and diarrhea have been seen. However, when the initial dose was reduced these effects were lessened (MacKay et al., 2000). Nitazoxanide (Navigator®, IDEXX Laboratories) has recently been approved by the FDA for treatment of horses with EPM.

Prevention

Prevention of EPM has been difficult because of the widespread distribution of the causative agents in many parts of the United Sates (MacKay et al., 2000). Though methods for effective control of the disease have not determined; it is imperative to eliminate known risk factors (MacKay et al., 2000).

Fort Dodge Animal Health has introduced an EPM vaccine that is used for the "vaccination of healthy horses as an aid in the prevention of neurologic disease caused by subsequent exposure to *Sarcocystis neurona*" (Fort Dodge Animal Health, 2001). At this time the vaccine is under conditional license from the United States Department of Agriculture (USDA). Full licensure will be granted following acceptable results from field efficacy or experimental infection studies currently in progress. The vaccine is made from *S. neurona* merozoites that are chemically inactivated. These chemically inactivated merozoites are then mixed with a non-alum adjuvant and administered.

Enzyme Linked Immunosorbent Assay

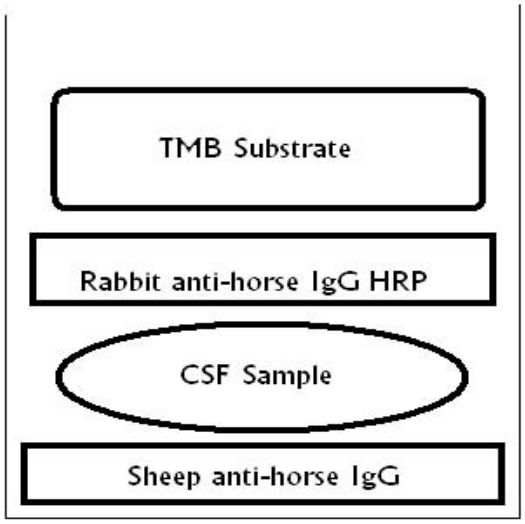
Enzyme linked immunosorbent assays (ELISA) provide highly sensitive and precise methods for estimation of biological parameters (Crowther, 1995). ELISA can be applied to various fields including the study of bacteria, fungi, protozoan and metazoan parasites. Through the use of ELISA, antigens and antibodies may be quantified in

various fluids and tissues. They are used both in research and in clinical settings and have been used for detection of and quantification of disease agents (i.e. parasite load, and antibodies) (Crowther, 1995).

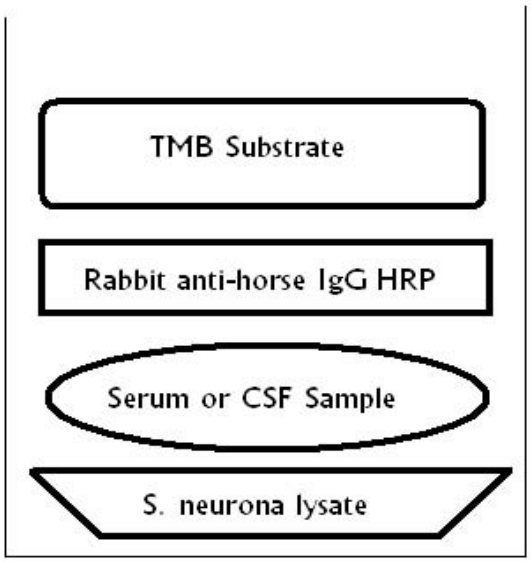
ELISA use passive absorption of one component to a solid phase (e.g., microtiter plate); subsequently reagents are successively added to the plate. Unreacted reagents can therefore be washed away. This technique allows for various ELISA reaction schemes. The basic principle of the assay relies on an enzyme attached to one of the agents (i.e. antibody) used in the assay. An enzyme substrate may then be added and yield a color change which can be read by the eye and/or quantified by spectrophotometer. Advantages of ELISAs include the large number of samples that may be run (a 96-well plate is used), requirement for very small volumes of test sample, ability to safely wash away unreacted reagents, and rapid results (read by eye or spectrophotometer) (Crowther, 1995).

There are four basic ELISA reaction schemes. These include the direct ELISA, indirect ELISA, sandwich ELISA, and competition ELISA. When beginning the use of an ELISA, the most appropriate reaction scheme must be selected. Things to consider when making this decision include the purpose of the assay, the reagents available, who will be performing the test, if the test will be used in other laboratories, and if a kit is required. The feasibility, validation, and standardization of the ELISA test are also important in the development of an assay (Crowther, 1995).

In our study, a direct sandwich ELISA and an indirect ELISA were developed. The direct sandwich ELISA, developed for the detection of equine total IgG from CSF samples, followed the following scheme:



An indirect ELISA was developed for the detection of *S. neurona* reactive IgG, which follows this scheme:



Goldmann-Witmer Coefficient

As briefly mentioned in the Immunoblot Test section above, there is normal traffic of proteins from blood into CSF through the blood-brain barrier (Cserr & Knopf., 1992). Thus, in the absence of CNS disease, all intrathecal IgG is plasma derived.

A specific CSF coefficient, or Goldmann-Witmer coefficient, has been used to differentiate antibodies in aqueous humor produced locally (in ocular tissues) from those produced systemically (Lappin et al., 1996b). According to the same principle, the specific CSF coefficient (C) should allow differentiation of the source of intrathecal antibodies. In a study done by Lappin and colleagues (1996b), it was assumed that C values greater than one were suggestive of local antibody production. In this study *Toxoplasma gondii*-specific and total IgG or total IgM were quantified in serum and aqueous humor by using ELISA and the C-values were then calculated using the following formula (Lappin et al., 1996b):

$$\frac{\textit{T. gondii}\ \text{IgM or IgG}_{\text{aqueous}}}{\textit{T. gondii}\ \text{IgM or IgG}_{\text{serum}}} \times \frac{\text{Total IgM or IgG}_{\text{serum}}}{\text{Total IgM or IgG}_{\text{aqueous}}}$$

A C-value greater than one was considered suggestive of local production of *T. gondii*-specific antibodies in aqueous humor.

Importance

The short-term objectives of this work were to develop ELISAs for total and *S. neurona*-reactive IgG. The development of these assays will lead to the development of a specific CSF coefficient as described above. The use of such a test may allow the identification of horses with *S. neurona* infection of the CNS and reduce or eliminate the numerous false-positives reported; thus allowing for accurate diagnosis of the disease and

reduction in inappropriate treatment costs. This will be a valuable tool and advancement in the study of EPM.

CHAPTER 3
MATERIALS AND METHODS

This study was conducted at the University of Florida, College of Veterinary Medicine in Gainesville, Florida. Analyses were conducted in the veterinary academic building (VAB) in room V2-242.

Development of *Sarcocystis neurona* ELISA

Experimental Animal

A horse obtained from Bayer Corporation's research herd (Shawnee Mission, KS) was used in this study. The horse was identified by halter tag number 866. The horse was fed only a complete pelleted ration (Purina Horse Chow 100; Purina Mills, LLC, St. Louis, Missouri) and had free choice water. The pasture in which the horse was kept was adapted to prevent access by opossums (Cutler, 1999). Briefly, the pasture was surrounded by welded-wire-mesh fence that was partially buried below the ground surface and had 2 strands of pulsed-electric wire on the outer surface.

Horse Pre-bleed

Approximately two liters of blood were collected by jugular venipuncture into polypropylene bottles (Nalge Nunc International, Rochester, NY). The bottles were kept at 37 C for 4 hours then at 4 C overnight to allow clot formation and retraction. Serum was harvested by centrifugation at 793 x *g* for 30 minutes and stored in aliquots at -80 C. An aliquot of serum was sent to both Neogen (Lexington, KY) and Equine Biodiagnostics (EBI) (Lexington, KY) for EPM immunoblot testing.

Sarcocystis neurona Isolate Culture

All cell culture procedures were performed using sterile technique in a laminar flow hood (Baker Company, Inc., Sanford, Maine).

Bovine turbinate cells (ATCC, CRL-1390, Manassas, VA) were chosen as the host cell for *S. neurona* culture. Bovine turbinate (BT) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.01 M HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum and kept at 37 C at 5% CO_2 in air. BT cells were seeded into tissue culture flasks and harvested by trypsinization and passed again when monolayers reached approximately 95% confluency.

A *Sarcocystis neurona* isolate (named WSU-1) was received as a gift from Dr. Maureen Long. This isolate has been described previously (Long et al., 2002). Briefly, this isolate was isolated from the blood of a 5-month old foal with sever combined immunodeficiency. The isolate was identified as *Sarcocystis neurona* based on genetic, serologic, and morphologic methods (Long et al., 2002). *Sarcocystis neurona* merozoites were added to flasks of confluent BT cells. Cultures were fed every two to three days by aspirating three-fourths of the culture supernate then replacing it with fresh culture medium. After 7-14 days, most BT cells had lysed as a result of intense shizogony and large numbers of merozoites could be collected free of BT cells. This spent medium was collected into 50 ml centrifuge tubes (Sarstedt Inc., Newton, NC), and then merozoites were enriched and used for horse inoculation as described below.

Sarcocystis neurona Merozoite Purification and Immunization

All purification steps below, except for counting, were performed using sterile technique in a laminar flow hood.

The culture supernates containing *S. neurona* merozoites were pooled and depleted of cell debris by passage through a 24-gauge hypodermic needle and centrifuged at 18.74 x g for five minutes. Merozoites were then pelleted by centrifugation at 388 x g for 20 minutes, re-suspended in phosphate buffered saline solution (PBSS) and counted. The merozoite suspension was carefully layered over ficoll-hipaque (SG 1.070; Optiprep®; Sigma-Aldrich, St. Louis, MO) and centrifuged at 400 x *g* for 45 minutes. The pellet, containing purified merozoites, was then resuspended in PBSS and washed by repeated cycles of centrifugation (507 x *g* for 10 minutes) and aspiration.

Once the merozoites were washed repeatedly, they were used for immunization of the experimental horse, for production of immune serum, or used below for immunoassay. For immunization the merozoites were prepared fresh on that day and after the final wash were suspended in 5 ml of PBSS and counted using a hemacytometer. On days 0, 14, and 28 horse #866 was inoculated intramuscularly and on day 42 was inoculated intravenously with at least 10^8 merozoites/dose. Two weeks after this final inoculation, 2 liters of blood was collected and serum was harvested and stored. A sample was sent to both Neogen, Inc. and EBI for immunoblot testing. This serum was then referred to as *immune* serum and used, as described below, for the *S. neurona* ELISA.

Extraction of *Sarcocystis neurona* Merozoites for Use in Immunoassay

Once the merozoites were purified as described above they were further processed to obtain antigen for use in the *S. neurona* ELISA. After the final wash of the merozoites in PBSS, the pellet was weighed after aspiration of supernate. To do this, an empty conical tube of the same size was weighed and then the conical tube with the pellet was weighed. The difference between the two tubes was taken and a pellet weight obtained.

A protein extraction reagent (Yeast Protein Extraction Reagent; Y-PER™; Pierce Biotechnology Inc., Rockford, IL) was mixed with the pellet at the rate of 2.5 ml per gram of cell pellet and then mixed by gentle vortexing for 20 minutes. At the end of the vortex step, cell debris was removed by centrifugation at 13,000 x *g* for ten minutes and the protein concentration of the lysate was determined by the micro-BCA Method (Microtiter Plate Protocol, Pierce Biotechnology Inc., Rockford, IL).

Equine CSF Total IgG Enzyme Linked Immunosorbent Assay

Assay Optimization

A sandwich ELISA was developed to quantify IgG in equine CSF samples. A checkerboard experiment was performed to determine optimal concentrations of antibodies. Affinity purified sheep anti-horse IgG-heavy and light chains (Bethyl Laboratories, Inc., Montgomery, TX) was used as the coating antibody, horse reference serum (Bethyl Laboratories, Inc., Montgomery, TX) as the standard, and whole molecule horseradish peroxidase-conjugated rabbit anti-horse IgG (Sigma-Aldrich, St. Louis, MO) as the detecting antibody. For the checkerboard assay, the sheep anti-horse IgG was diluted in coating buffer (carbonate/bicarbonate buffer, pH 9.6) starting at 1:1,000 (in column 1) and diluted 2-fold across an Immulon 2HB microtitration plate (Thermo Labsystems, Franklin, MA) plate to a final dilution of 1:1,024,000 (in column 12). After incubation at 4 C for three hours, each well of the plate was washed four times with approximately 200 µl of PBSS-0.5% tween 20 (PBSST) using a Nunc-Immuno washer (Nalge Nunc International, Rochester, NY). Next, blocking buffer consisting of 150 µl 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBSST was added to each of the wells of the plate. After 20 hours incubation at room temperature, 100 µl of equine reference serum was added to wells for an additional 1 hour at room temperature,

then the plate was washed again four times with PBSST. One hundred microliter amounts of serial 2-fold dilutions of secondary antibody (1:1,000 to 1:128,000 in PBSST) were then added to the wells and incubated for an hour at room temperature. Next, the plate was washed four times and 100 µl of 3,3',5,5' tetramethylbenzidine (TMB) (SureBlue KPL, Inc., Gaithersburg, MD) peroxidase substrate was added to all wells. Thirty minutes later the reaction was stopped by addition of 100 µl of 1% HCl to all wells. The plate was then read at 450 nm using a Bio-Tek plate reader (Bio-Tek Instruments, Winooski, VT).

On the basis of the above results, the standard protocol for the CSF total IgG ELISA was established, following the above incubation times and temperatures. For the standard procedure, the steps above were followed using the sheep anti-horse IgG diluted 1:8,000 in coating buffer and the rabbit anti-horse IgG-HRP diluted 1:45,000 in PBSST. The standard curve, using equine reference serum, was run at dilutions from 1:80,000 to 1:2,560,000 in PBSST.

Optimization of Plate Type and Blocking Protocol

An experiment was conducted to determine the optimal plate type for use in this assay. The above protocol for the equine total IgG ELISA was used while testing various plates. Plates included in this trial included Immulon 1B (Thermo Labsystems, Franklin, MA), Immulon 2B (Thermo Labsystems, Franklin, MA), Costar (Corning Inc., Acton, MA), and Maxisorp (Nalge Nunc International, Rochester, NY). The plate that was chosen was one which yielded the lowest non-specific binding of sample to the plate. This plate also yielded a smooth standard curve.

An experiment to determine the most efficient blocker was also performed. The blockers included in the experiment were 3% BSA, 1% Blotto in PBSST, and 5% Blotto

in PBSST. Three Immulon 2HB plates were run in parallel and each of the aforementioned blocking solutions was used following the equine total IgG ELISA protocol above. The blocking solution was chosen on the basis of providing the least amount of non-specific binding.

Inter- and Intra-Assay Optimization

The assay was performed five times in parallel to determine intra-assay repeatability and was performed on five successive days to determine inter-assay repeatability.

Quantification of CSF IgG

A best-fit standard curve for log reference serum dilution versus OD_{450} was generated on each plate by using curve-fitting software (SigmaPlot 8.0; SPSS, Inc., Chicago, IL). IgG concentration in undiluted reference serum was first determined by radial immunodiffusion (VMRD, Inc., Pullman, WA). IgG in CSF samples on that plate were then quantified by interpolation into the standard curve.

Equine Serum Total IgG Quantification

For determination of IgG in serum samples, radial immunodiffusion (RID) assays were performed by Neogen Corporation, Lexington, Kentucky.

ELISA for *Sarcocystis neurona* IgG

S. neurona Optimization

An ELISA was developed for the detection of *S. neurona*-specific IgG. The first step in the development of the assay involved determining optimal concentrations and dilutions of antigen, samples, and antibodies. *Sarcocystis neurona* lysate, prepared as described above, was used as the coating antigen, serum from the *S. neurona* inoculated horse (referred to as *immune* serum) was used for generation of a standard curve, and

horseradish peroxidase-labeled rabbit anti-horse IgG (Sigma-Aldrich, St. Louis, MO) was the detecting antibody. The substrate used was 3,3',5,5' tetramethylbenzidine (TMB) (SureBlue KPL, Inc., Gaithersburg, MD).

First, a checkerboard experiment was conducted to determine optimal concentrations of the coating antigen and the detecting antibody. The lysate was diluted (serial 2-fold) in coating buffer (carbonate/bicarbonate buffer, pH 9.6) from 8 µg to 0.0625 µg down an Immulon 2HB microtitration plate (Thermo Labsystems, Franklin, MA) plate. After incubation for 20 hours at 4 C, the coated plate was washed four times with PBSST. After washing, 150 µl of 1% BSA in PBSST was added to wells and the plate was incubated for two hours at room temperature. The immune serum was then added to the entire plate at a dilution of 1:5,000 in PBSST and allowed to incubate for one hour at room temperature. The plate was then washed four times with PBSST and 100 µl of serial 2-fold dilutions (from 1:1,000 to 1:32,000) of rabbit anti-horse IgG HRP in PBSST were added to wells and allowed to incubate at room temperature. The plate was then washed after one hour of incubation and 100 µl TMB (SureBlue KPL, Inc., Gaithersburg, MD) was added to wells. The plate was left in the dark at room temperature for 15 minutes and the reaction was then stopped by the addition of 100 µl of 1% HCl to wells. The plate was then read at 450 nm using a Bio-Tek plate reader (Bio-Tek Instruments, Winooski, VT).

The range of the standard curve was determined next by following the above procedure and using the *S. neurona* lysate diluted to 0.5 µg/ml in coating buffer (carbonate/bicarbonate buffer, pH 9.6) and the rabbit anti-horse IgG-HRP diluted 1:2,000 in PBSST. After the 2 hour blocking step, immune serum diluted in PBSST was added

across the plate (range 1:10 to $1:10^{12}$) and allowed to incubate for one hour at room temperature. Once the plate was read, the standard curve dilutions were determined.

On the basis of the above results, the standard protocol for the *S. neurona* ELISA was established, following the above incubation times and temperatures. For the standard procedure, the steps above were followed using the *S. neurona* lysate diluted to 0.5 µg in coating buffer and the rabbit anti-horse IgG-HRP diluted 1:2,000 in PBSST. The standard curve, using *immune* serum was run at dilutions from 1:800 to 1:25,600 in PBSST.

Optimization of Plate Type and Blocking Protocol

These experiments were identical to that described above for optimization of the total IgG assay.

Inter and Intra Assay Repeatability

The assay was performed five times in parallel to determine intra-assay repeatability and was performed on five successive days to determine inter-assay repeatability.

Quantification of anti-*S. neurona* IgG – ELISA Units/ml

A standard curve for relative *S. neurona* IgG concentration versus OD_{450} was generated from each plate and a line was fitted by linear regression. The *S. neurona* IgG concentration of samples on that plate were then determined by interpolation into the standard curve. These values were expressed as ELISA units (EU) with undiluted immune serum ascribed a nominal value of 100,000 EU/ml.

Calculation of CSF Coefficient

CSF coefficients were calculated using a modification of the Goldmann-Witmer coefficient (Lappin et al., 1996b), according to the following formula:

$$\frac{\textit{S. neurona}\ \text{IgG}_{\text{CSF}}}{\textit{S. neurona}\ \text{IgG}_{\text{serum}}} \times \frac{\text{Total IgG}_{\text{serum}}}{\text{Total IgG}_{\text{CSF}}}$$

Sample Description

The samples used in the assays were run blinded and were generated during previous studies that are not a part of this work. The samples came from 2 different experiments, which will be described as experiments 1 and 2, below. In experiment 1 there were 4 different groups as follows: (group 1) 4 horses challenged with *S. neurona* sporocysts and not given treatment; (group 2) 5 horses challenged with *S. neurona* sporocysts and given ponazuril every 7 days; (group 3) 5 horses challenged with *S. neurona* sporocysts and given ponazuril every 14 days; (group 4) 4 horses neither challenged nor treated. In experiment 2 there were 3 different groups as follows: (group 5) 4 horses challenged with *S. neurona* sporocysts and given dexamethasone; (group 6) 5 horses neither challenged nor treated; (group 7) 4 horses were challenged with *S. neurona* sporocysts and received no treatment. In experiment 1, horses were administered a single intragastric inoculum of 5×10^5 sporocysts and given ponazuril paste (150 mg/ml) orally at 20 mg/kg at the specified interval, beginning 7 days after challenge. In experiment 2, horses were given an intragastric inoculum of 4×10^5 sporocysts daily for 7 days (total 2.8×10^6) and treated daily IM with 0.1 mg/kg dexamethasone. In both experiments, controls were given only water vehicle intragastrically and/or no other treatment. The "before" blood and CSF samples were taken at day 0 of the experiment, immediately prior to sporocyst challenge; and the "after" samples were taken at the time each horse was euthanized 78 - 157 days after challenge.

Statistical Analyses

The data from the experimental samples, as described above, did not meet ANOVA assumptions for homogeneous variance or normal distribution, so non-parametric tests of significance were used for all analyses. Effects of time (i.e., difference between "after" value and "before" value) were explored *within* groups by Wilcoxon signed-rank tests. Effects of treatment group on various values were examined by Kruskal-Wallis tests. When a significant effect of was found, differences between pairs of groups were explored by Mann-Whitney U tests. Differences between groups defined by western blot results were examined by Mann-Whitney U (2-level western blot classification) or Kruskal-Wallis (3-level western blot classification). Samples from the 2 experiments were grouped together depending on their immunoblot results. In the 2-level classification, the groups were designated as negative (including nonspecific results) or positive (including weak positive results); whereas, in the 3-level classification, the groups were designated as negative, intermediate (weak positive or non-specific), and positive. For all analyses, significance was ascribed to P #0.05. A commercial statistical software package (SPSS 12.0 for Windows; SPSS Inc, Chicago, IL) was used to perform analyses.

Clinical Sample Analyses

Thirty-seven clinical samples were obtained from the University of Florida, College of Veterinary Medicine, Clinical Microbiology Laboratory. On the basis of their diagnosis upon admission to the college, these samples were divided into three groups: EPM, Non-EPM, and undiagnosed. Differences among these groups were explored using the Kruskal-Wallis test. When a significant effect was found for a variable a Tukey box blot was generated. Additional analysis was performed comparing these 3 clinical groups

along with 2 experimental groups from above (all experimental horses before challenge and the experimental control groups).

CHAPTER 4
RESULTS

Immunoblot Testing Results

The serum sample obtained prior to inoculation of #866 tested non-specific at Neogen, Inc. and negative at EBI. The serum sample taken 2 weeks after the inoculation series tested positive at both labs by immunoblot analysis. This signified seroconversion had taken place and we could use these serums for immune or pre-immune serum standards on the *S. neurona* ELISA.

Sarcocystis neurona Lysate Stock Concentration

The protein concentration of the *S. neurona* lysate was determined to be 5.6 mg/ml.

Equine CSF Total IgG ELISA

On the basis of the results of the checkerboard experiment for the equine CSF total IgG ELISA, it was determined that the optimal antibody dilutions were 1:8,000 and 1:45,000 for the coating antibody (sheep anti-horse IgG) and secondary antibody (rabbit anti-horse-HRP) respectively. The reference equine serum, used as the standard in the equine CSF total IgG ELISA, was used at serial 2-fold dilutions ranging from 1:80,000 to 1:2,560,000. CSF samples were diluted to 1:1,000 and 1:10,000 for assay. The best blocking solution was 3% bovine serum albumin and the best-performing plate was Immulon 2 HB. The coefficient of variance (CV) for repeated assay of samples was 9.1 $\pm$ 1.4% ($\bar{x} \pm$ sem) within assays and 10.7 $\pm$ 1.9% between assays.

Tables and figures 4-1 and 4-2 below show the effects of *S. neurona* challenge (i.e., time) and treatment with ponazuril or dexamethasone on CSF total IgG. There was no significant effect of time or treatment group on IgG concentration in either experiment.

Table 4-1. Effect of challenge and ponazuril on CSF IgG concentration ($\bar{x} \pm$ sem).

group	*N*	Challenge	Treatment	IgG concentration (mg/dl) before	IgG concentration (mg/dl) after	*P*-value*
1	4	+	-	17.5±7.6	19.6±8.8	0.47
2	5	+	+(7)	20.6±3.4	20.6±3.4	0.69
3	5	+	+(14)	20.3±5.4	20.3±5.4	0.35
4	4	-	-	14.5±3.0	14.5±3.0	0.14

* *P*-value for difference between before and after values.

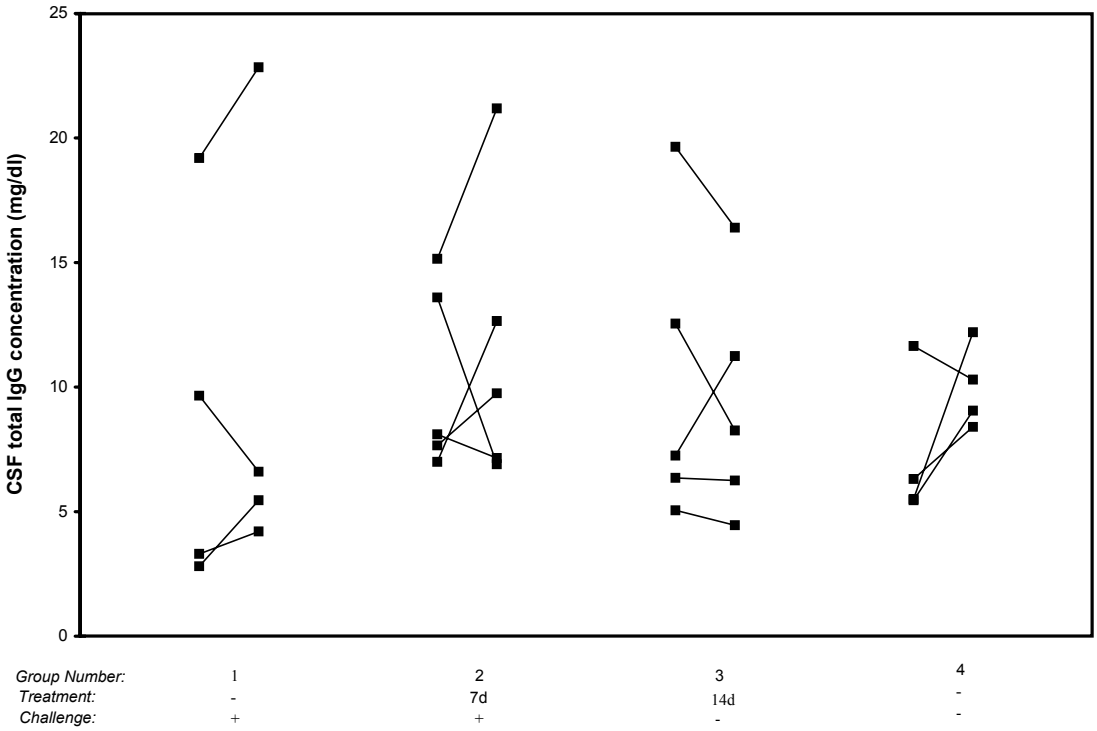


Figure 4-1. Effect of *S. neurona* challenge and intermittent ponazuril treatment on IgG concentration of CSF. For each group, experimental conditions (challenge and treatment) are shown below data points for that group. For each experimental horse, a line connects the values obtained before and after challenge.

Table 4-2. Effect of challenge and dexamethasone on CSF IgG concentration ($\bar{x} \pm$ sem).

group	*N*	Challenge	Treatment	IgG concentration (mg/dl) before	IgG concentration (mg/dl) after	*P*-value*
5	4	+	+	13.0±3.5	16.4±3.4	0.07
6	5	-	-	7.9±1.6	5.1±1.6	0.23
7	4	+	-	10.2±3.8	7.9±2.5	0.47

**P*-value for difference between before and after values.

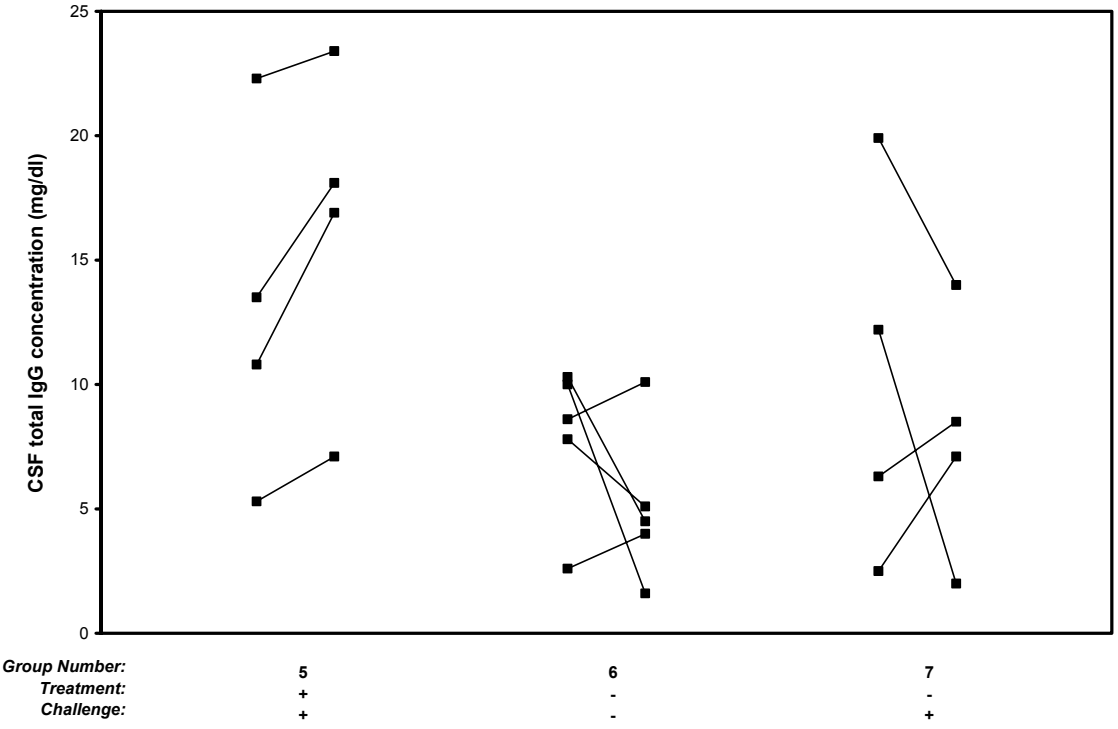


Figure 4-2. Effect of *S. neurona* challenge and daily dexamethasone treatment on IgG concentration of CSF. For each group, experimental conditions (challenge and treatment) are shown below data points for that group. For each experimental horse, a line connects the values obtained before and after challenge.

Equine Serum Total IgG RID

Tables and figures 4-3 and 4-4 below show the effects of *S. neurona* challenge (time) and treatment with ponazuril or dexamethasone on serum IgG concentration. There was no significant effect of time or treatment group on IgG concentration in either experiment.

There was a significant effect ($P = 0.04$) of treatment group on *change* (i.e., *after* value minus *before* value) in IgG concentration over the period of the experiment. Further pairwise analyses between groups revealed significant effect of dexamethasone treatment on *S. neurona*-challenged horses (Table 4.4). No other significant effect of time or treatment group was discovered.

Table 4-3. Effect of challenge and ponazuril on serum IgG concentration ($\bar{x} \pm$ sem).

				IgG concentration (mg/dl)		
group	*N*	Challenge	Treatment	before	after	*P*-value*
1	4	+	-	2,954±350.3	3,028.9±382.6	0.47
2	5	+	+(7)	3,202.5±217.6	3,121.1±269.6	0.89
3	5	+	+(14)	3,469.6±357.6	3,104.4±137.6	0.35
4	4	-	-	3,369.1±556.3	3,134.8±200.9	0.72

* *P*-value for difference between before and after values.

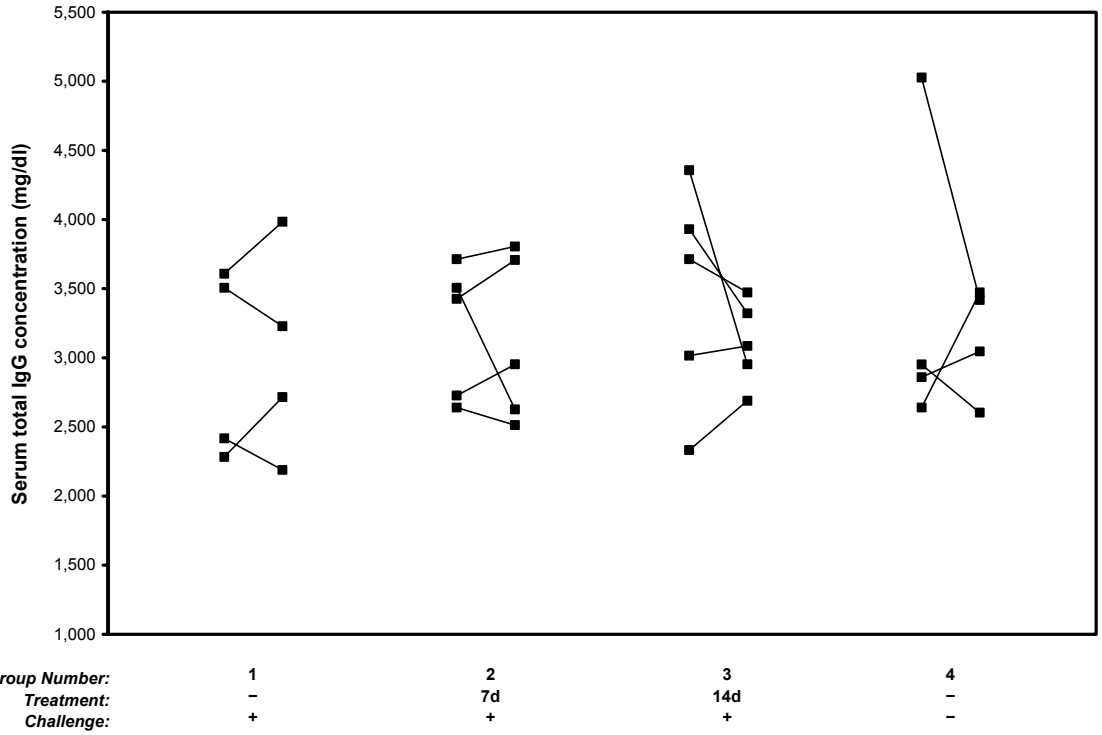


Figure 4-3. Effect of *S. neurona* challenge and intermittent ponazuril treatment on serum IgG concentration. Details of presentation are explained in the legend to figure 4-1.

Table 4-4. Effect of challenge and dexamethasone on serum IgG concentration ($\bar{x}$ ± sem).

group	*N*	challenge	treatment	IgG concentration (mg/dl) before	IgG concentration (mg/dl) after†	*P-value**
5	4	+	+	4,294.5±292.7	3,378.0±319.0[a]	0.14
6	5	-	-	3,298.6±781.7	3,616.0±1034.6[a,c]	0.50
7	4	+	-	4,092.2±520.4	5,101.2±617.6[b,c]	0.07

* *P-value* for difference between before and after values.

† Superscripted letters are used to designate pairwise differences in *change* of IgG concentration (*after* minus *before*). Pairs of values that do not share the same superscripted letter are significantly different.

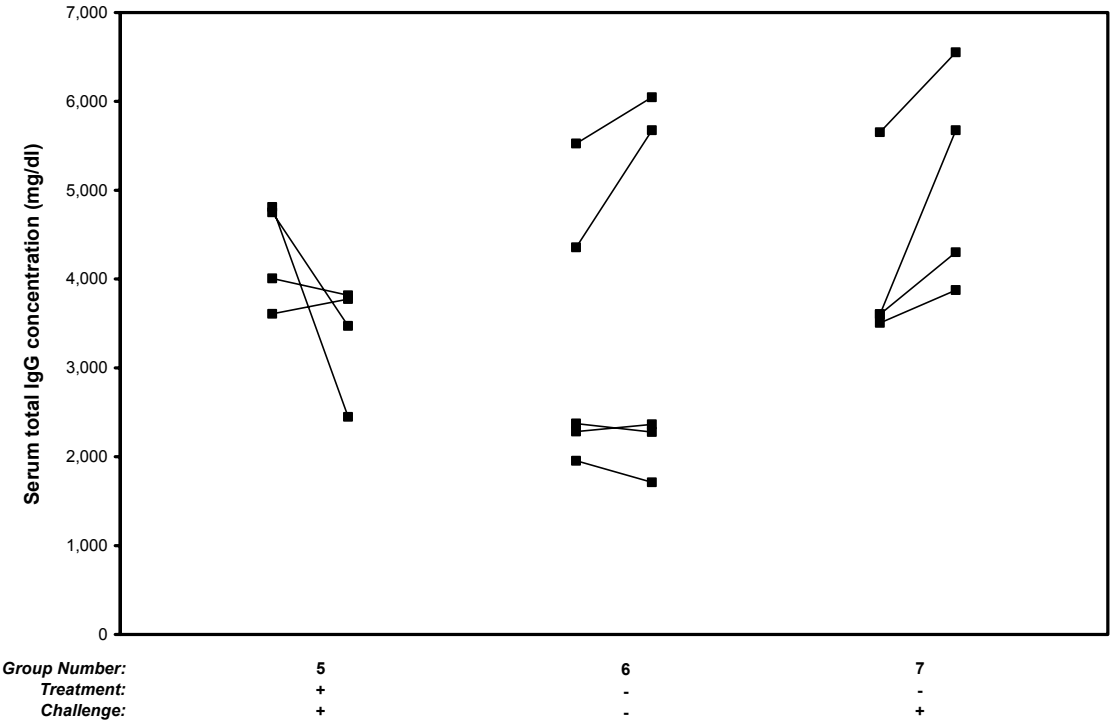


Figure 4-4. Effect of *S. neurona* challenge and dexamethasone treatment on serum IgG concentration. Details of presentation are explained in the legend to figure 4-2.

Sarcocystis neurona ELISA

On the basis of the checkerboard assay for the *S. neurona* ELISA, it was determined that, for experimental assays, *S. neurona* lysate should be diluted to 0.5 µg/ml, and immune serum for standard curve generation should be run at dilutions ranging from 1:800 to 1:25,600. For assay, test serum samples were to be diluted 1:200

and 1:2,000 and CSF samples 1:2 and 1:20. The best blocking solution was 1% BSA and the best performing plate was Immulon 2HB. The coefficient of variance (CV) for repeated assay of samples was 9.3 ± 2.3% ($\bar{x}$ ± sem) within assays and 16.0 ± 4.0% between assays.

Tables and figures 4-5 and 4-6 below show the effects of *S. neurona* challenge (time) and treatment with ponazuril or dexamethasone on serum *S. neurona* titer. In the first experiment, there was a substantial *increase* in titer in all groups which were given *S. neurona*. This effect was significant within groups 2 and 3. Furthermore, there was a significant effect ($P = 0.01$) of treatment group on magnitude of titer increase. *Post hoc* pairwise analyses between groups showed that the increase in titer was significantly greater in all challenged groups than it was in the control group (group 4). Mean titers increased by more than 100,000 EU/ml in each of the challenged groups in experiment 2 although this effect was not significant. There was significant effect ($P = 0.02$) of treatment group on magnitude of titer increase during the experiment. Pairwise analyses of this effect showed that the increase in titer was significantly greater in the 2 challenged groups than it was in the control group (group 2).

Table 4-5. Effect of challenge and ponazuril on serum *S. neurona* titer ($\bar{x}$ ± sem).

Group	*N*	challenge	treatment	*S. neurona* titer (EU/ml)		*P*-value*
				before	after†	
1	4	+	-	2,811.0±736.3	53,510.1±18,410.8[a]	0.07
2	5	+	(+)7	3,325.5±1,528.6	29,057.6±14,958.7[a]	0.04
3	5	+	(+)14	6,841.0±3,072.4	82,153.7±32,166.7[a]	0.04
4	4	-	-	2,010.1±301.5	1,737.1±245.0[b]	0.14

* *P*-value for difference between before and after values.

† Superscripted letters are used to designate pairwise differences in *change* of IgG concentration (*after* minus *before*). Pairs of values that do not share the same superscripted letter are significantly different.

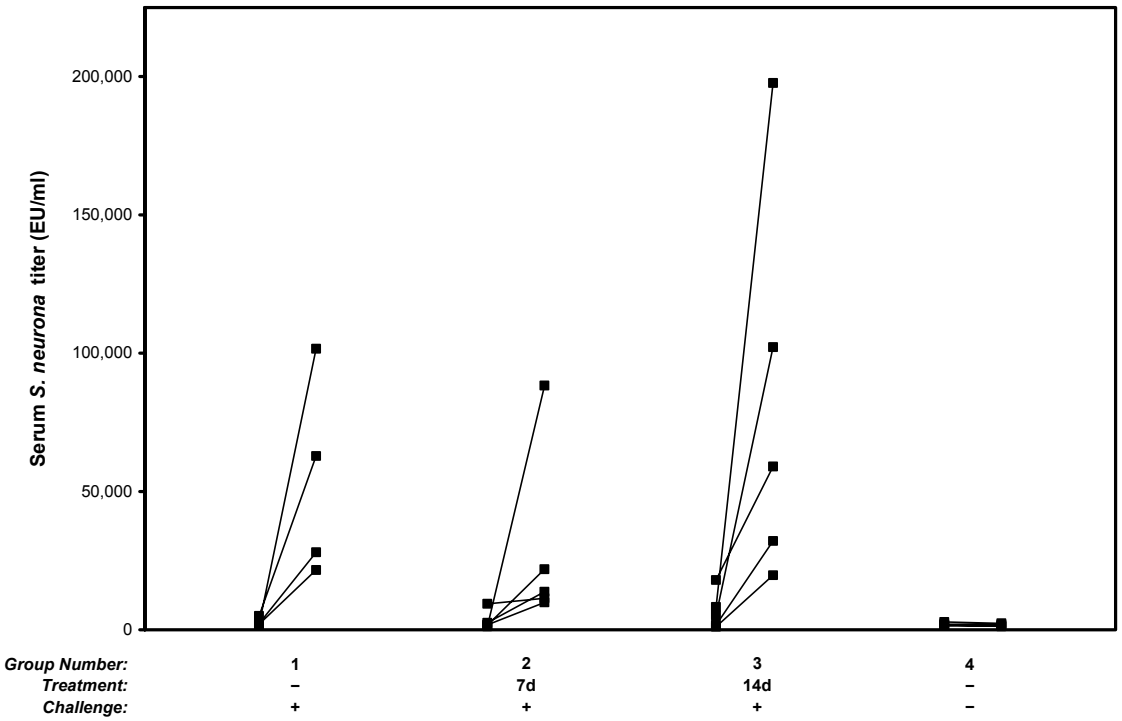


Figure 4-5. Effect of *S. neurona* challenge and intermittent ponazuril treatment on serum *S. neurona* titer. Details of presentation are explained in the legend to figure 4-1.

Table 4-6. Effect of challenge and dexamethasone on serum *S. neurona* titer ($\bar{x}$ ± sem).

				S. neurona titer (EU/ml)		
group	*N*	challenge	Treatment	before	after[†]	*P*-value[*]
5	4	+	+	2,060.3±237.9	130,4413.4±480,630.2[a]	0.07
6	5	-	-	10,183.3±6,508.1	37,766.9±28,829.5[b]	0.35
7	4	+	-	2,286.3±1,072.9	423,403.2±233,556.7[a]	0.07

* *P*-value for difference between before and after values.

[†] Superscripted letters are used to designate pairwise differences in *change* of IgG concentration (*after* minus *before*). Pairs of values that do not share the same superscripted letter are significantly different.

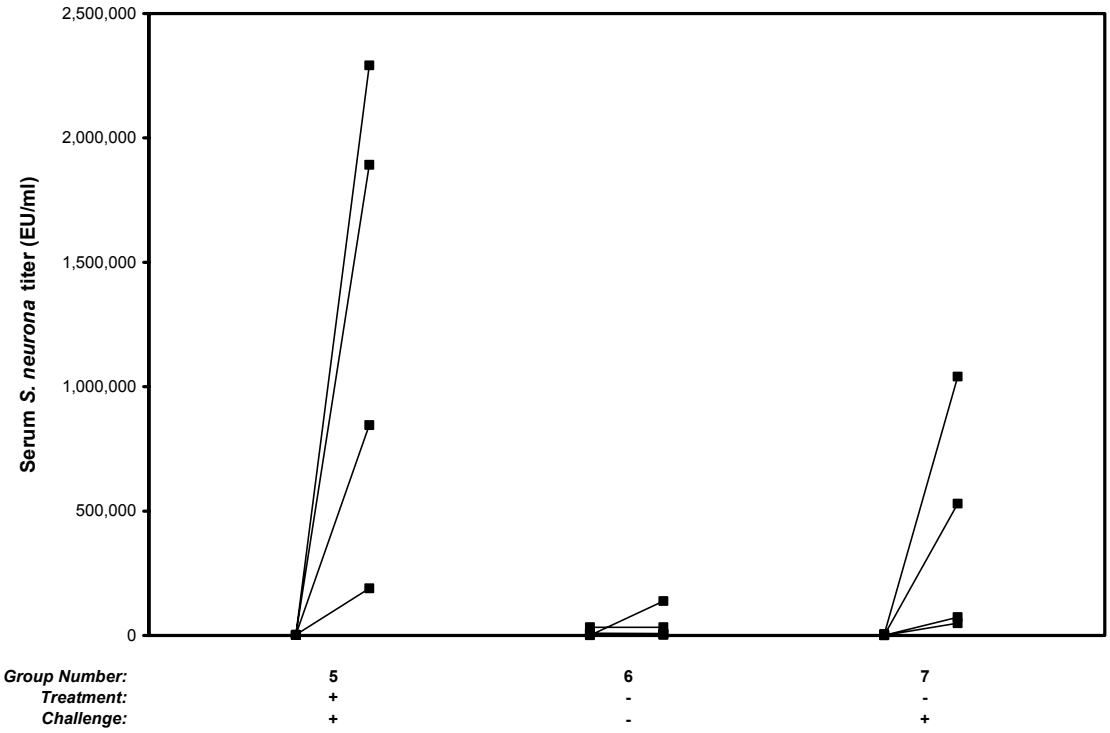


Figure 4-6. Effect of *S. neurona* challenge and dexamethasone treatment on serum *S. neurona* titer. Details of presentation are explained in the legend to figure 4-2.

Tables and figures 4-7 and 4-8 below show the effects of *S. neurona* challenge (time) and treatment with ponazuril or dexamethasone on *S. neurona* titer in CSF. In the first experiment, there was a greater than 10-fold *increase* in titer in all groups which were given *S. neurona*. This effect was significant within groups 2 and 3. As was the case in serum, there also was a significant effect ($P = 0.01$) of treatment group on titer increase in CSF. *Post hoc* pairwise analyses showed that the increase in titer over the experiment was significantly greater in all challenged groups than it was in the control group (group 4). Mean CSF titers increased by more than 2,000 EU/ml in each of the challenged groups in experiment 2, although this effect was not significant. There was significant effect ($P = 0.02$) of treatment group on titer increase, however. Pairwise

analysis of this effect revealed that the increase in titer was significantly greater in the 2 challenged groups than it was in the control group (group 2).

Table 4-7. Effect of challenge and ponazuril on CSF *S. neurona* titer ($\bar{x}$ ± sem).

group	*N*	challenge	treatment	*S. neurona* titer (EU/ml)		*P-value*[*]
				before	after[†]	
1	4	+	-	7.8±0	346.7±184.2[a]	0.07
2	5	+	+(7)	8.6±0.78	129.8±52.7[a]	0.04
3	5	+	+(14)	10.8±1.6	557.7±296[a]	0.04
4	4	-	-	9±1.2	8.8±1[b]	0.32

* *P-value* for difference between before and after values.

† Superscripted letters are used to designate pairwise differences in *change* of IgG concentration (*after* minus *before*). Pairs of values that do not share the same superscripted letter are significantly different.

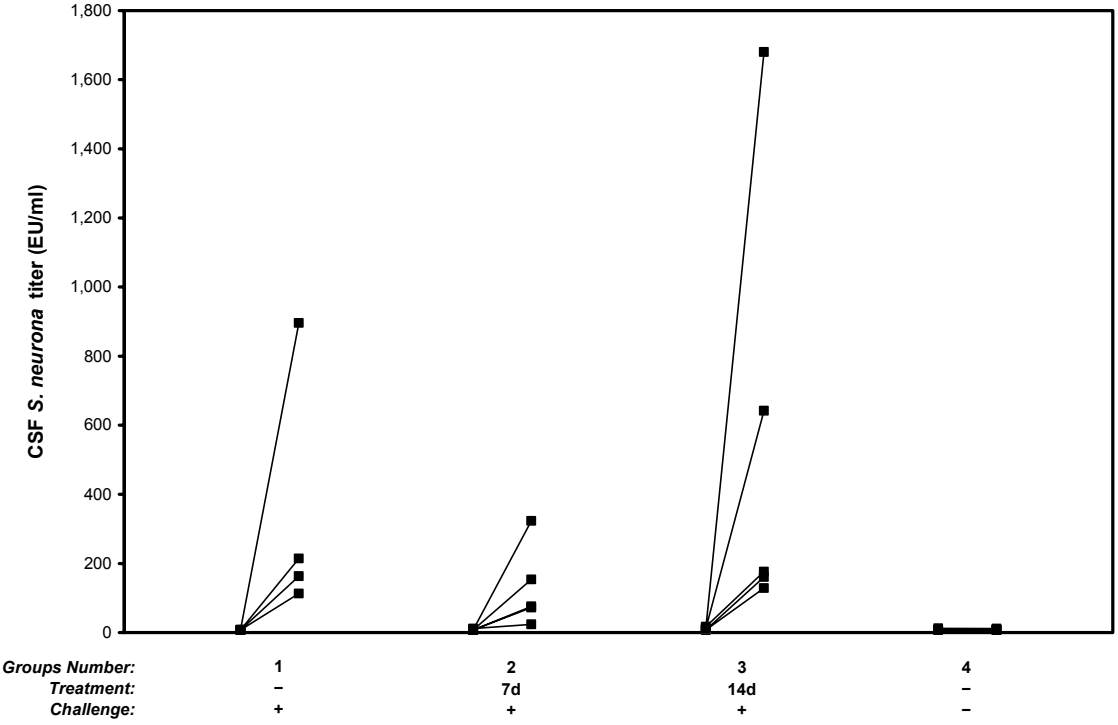


Figure 4-7. Effect of *S. neurona* challenge and intermittent ponazuril treatment on *S. neurona* titer in CSF. Details of presentation are explained in the legend to figure 4-1.

Table 4-8. Effect of challenge and dexamethasone on CSF *S. neurona* titer ($\bar{x}$ ± sem).

group	*N*	challenge	treatment	*S. neurona* titer (EU/ml)		*P*-value*
				before	after†	
5	4	+	+	7.8±0	10,177.5±4,084.6[a]	0.07
6	5	-	-	36.5±31.7	244.9±242.4[b]	1.00
7	4	+	-	7.8±0	2,542.4±1,606.4[a]	0.07

* *P*-value for difference between before and after values.

† Superscripted letters are used to designate pairwise differences in *change* of IgG concentration (*after* minus *before*). Pairs of values that do not share the same superscripted letter are significantly different.

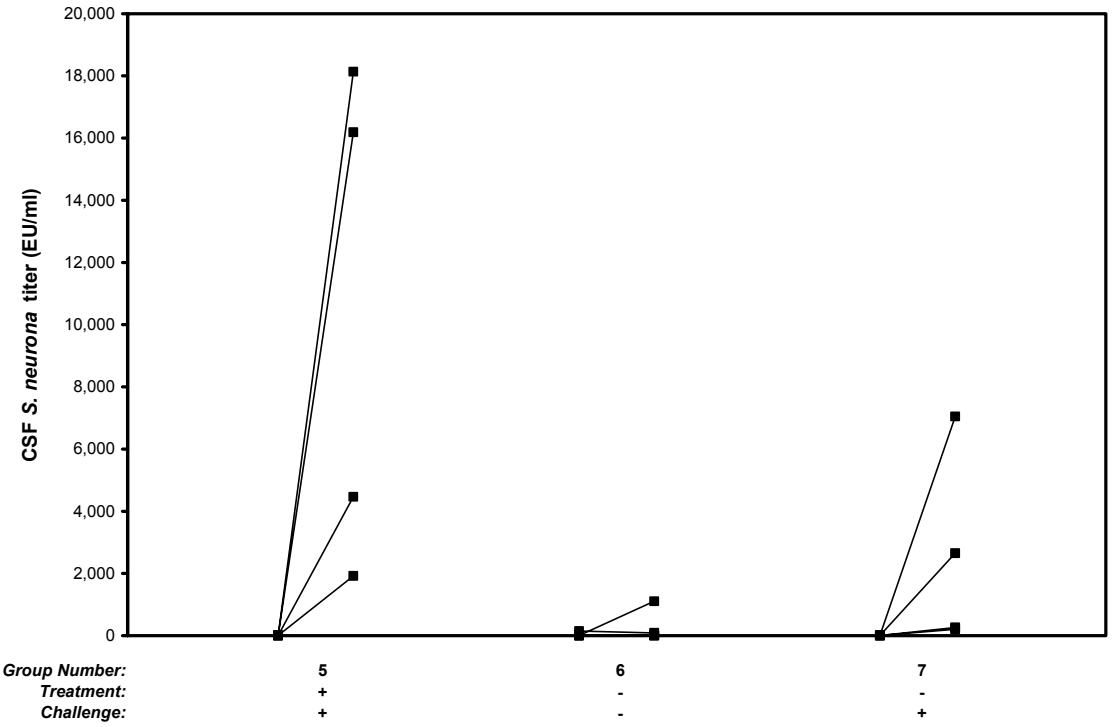


Figure 4-8. Effect of *S. neurona* challenge and dexamethasone treatment on *S. neurona* titer in CSF. Details of presentation are explained in the legend to figure 4-2.

Coefficients

Tables and figures 4-9 and 4-10 below show the effects of *S. neurona* challenge (time) and treatment with ponazuril or dexamethasone on *S. neurona* coefficients (C). Significant effect of time or treatment group was not found in either experiment. However, some C values were artificially high, for example in group 7 in experiment 2, due to high background in CSF samples.

Table 4-9. Effect of challenge and ponazuril on coefficient ($\bar{x}$ ± sem).

Group	*N*	challenge	treatment	Coefficient		*P*-value*
				Before	After	
1	4	+	-	1±0.35	1.1±0.16	0.47
2	5	+	+(7)	0.63±0.15	0.71±0.1	0.50
3	5	+	+(14)	0.71±0.32	1.1±0.27	0.23
4	4	-	-	1.1±0.09	0.82±0.06	0.07

* *P*-value for difference between before and after values.

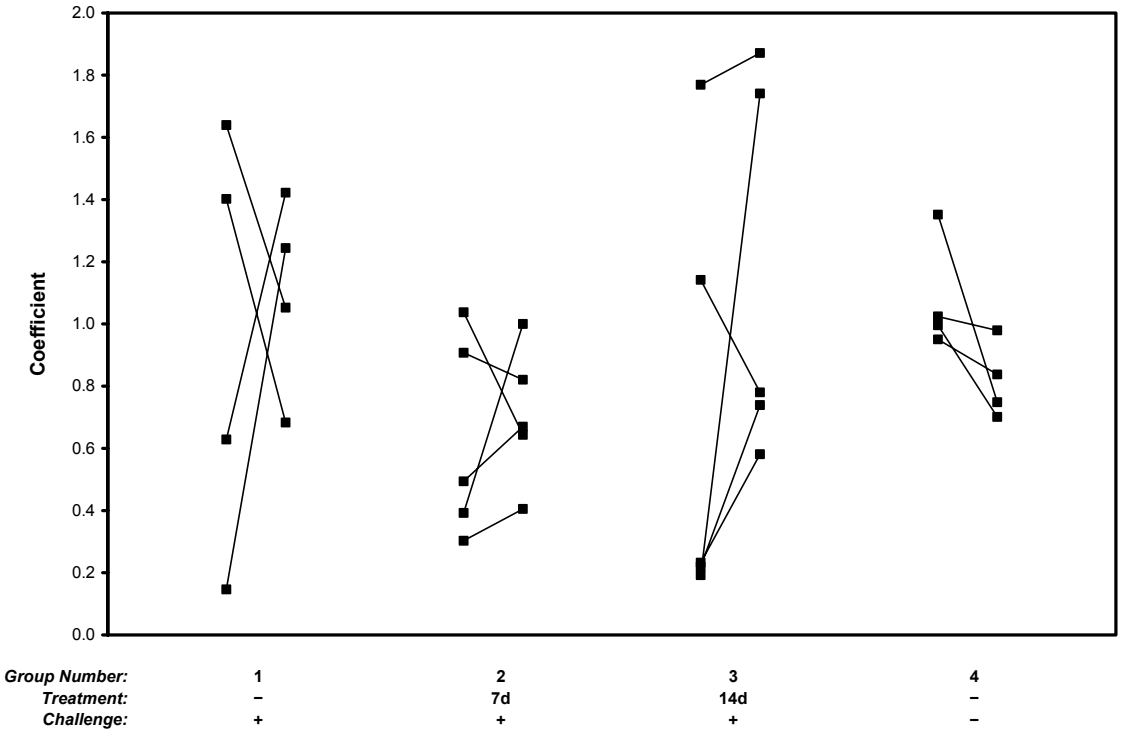


Figure 4-9. Effect of *S. neurona* challenge and intermittent ponazuril treatment on calculated coefficients. Details of presentation are explained in the legend to figure 4-1.

Table 4-10. Effect of challenge and dexamethasone on coefficient ($\bar{x}$ ± sem).

group	*N*	challenge	treatment	Coefficient		*P-value**
				before	After	
5	4	+	+	1.8±0.54	1.8±0.35	0.72
6	5	-	-	1.8±1	1.9±0.44	0.50
7	4	+	-	3.5±1.4	4.1±1	0.72

* *P*-value for difference between before and after values.

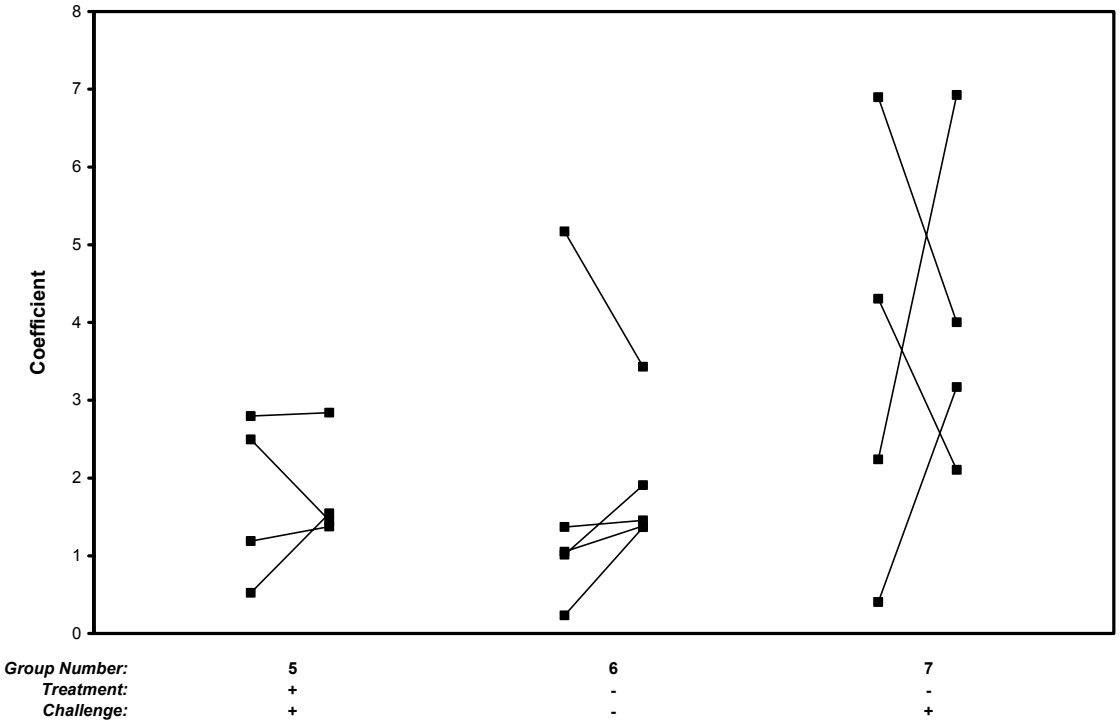


Figure 4-10 Effect of *S. neurona* challenge and dexamethasone treatment on calculated coefficients. Details of presentation are explained in the legend to figure 4-2.

Immunoblot Analysis

Immunoblots for *S. neurona* IgG were obtained for all samples in experiments 1 and 2. These immunoblots were performed by either Equine Biodiagnostics Incorporated (EBI, Lexington, KY) or Neogen Corporation (Lexington, KY). Results were classified according to both a 2-level (positive, negative) and a 3-level (positive, intermediate, negative) system. The criteria for these classification systems are given in Chapter 3, Materials and Methods.

In Table 4-11 are shown the results of grouping all serum values according to immunoblot result (2-level immunoblot classification). For serum *S. neurona* titer, SN ratio, and C mean values were significantly higher in the samples classified as immunoblot-positive than they were for those classified as negative.

Table 4-11. Effect of classification according to immunoblot result (positive or negative) on serum *S. neurona* and IgG parameters ($\bar{x}$ ± sem)

WB result	*N*	*S. neurona* titer (EU/ml)	SN ratio*	IgG conc (mg/dl)	IgG ratio†	C
negative	29	5736.2±1563.8	0.38±0.03	3,276.3±178.8	0.49±0.05	1.20±0.27
positive	33	237,855.1±93,240.9	0.57±0.06	3,680.5±195.6	0.43±0.05	1.81±0.24
all	62	129,283.3±51,457.7	0.48±0.04	3,491.5±135	0.46±0.04	1.52±0.18
P-value		<0.001	0.027	0.089	0.217	0.005

* *S. neurona* titer CSF/*S. neurona* titer serum (×100)
† IgG concentration CSF/IgG concentration serum (×100)
C = CSF coefficient; *N* = Number

In Table 4-12 are shown the results of grouping all CSF values according to immunoblot result (2-level immunoblot classification). For CSF *S. neurona* titer, SN ratio, C, and RQ, mean values were significantly higher in the samples classified as immunoblot-positive than they were for those classified as negative.

Table 4-12. Effect of classification according to immunoblot result (positive or negative) on CSF *S. neurona* and IgG parameters ($\bar{x}$ ± sem)

WB result	*N*	*S. neurona* titer (EU/ml)	SN ratio*	IgG conc (mg/dl)	IgG ratio†	C	RQ
Negative	38	24.9±9.2	0.37±0.03	15.3±1.5	0.46±0.04	1.17±0.21	0.00±0.00
Positive	24	2,351.1±992.9	0.65±0.07	15.5±2.3	0.46±0.07	2.08±0.3	19.38±3.96
All	62	925.4±406.1	0.48±0.04	15.4±1.3	0.46±0.04	1.52±0.18	7.50±1.94
P-value		<0.001	<0.001	0.644	0.549	0.001	<0.001

* *S. neurona* titer CSF/*S. neurona* titer serum (×100)
† IgG concentration CSF/IgG concentration serum (×100)
C = CSF coefficient; N = Number

In Table 4-13 are shown the results of grouping all serum values according to immunoblot result (3-level immunoblot classification). For serum *S. neurona* titer and SN ratio, there was a significant effect of grouping by immunoblot result.

Table 4-13. Effect of classification according to immunoblot result (positive, intermediate, or negative) on serum *S. neurona* and IgG parameters ($\bar{x}$ ± sem).

WB result	*N*	*S. neurona* titer (EU/ml)	SN ratio*	IgG conc (mg/dl)	IgG ratio†	C
negative	2	17,355.5±15,570.9	0.45±0.01	2,662.4±289.2	0.43±0.00	1.04±0.01
intermediate	31	4,859.6±1,151.0	0.35±0.03	3,483.3±203.4	0.45±0.05	1.23±0.25
positive	29	270,007.2±104,888.9	0.61±0.06	3,557.3±188.2	0.47±0.06	1.86±0.27
all	62	129,283.3±51,457.7	0.48±0.04	3,491.5±135.0	0.46±0.04	1.52±0.18
P-value		<0.001	0.002	0.319	0.99	0.052

* *S. neurona* titer CSF/*S. neurona* titer serum (×100)
† IgG concentration CSF/IgG concentration serum (×100)
C = CSF coefficient; *N* = Number

In Table 4-14 are shown the results of grouping all CSF values according to immunoblot result (3-level immunoblot classification). For CSF *S. neurona* titer, SN ratio, C, and RQ, there was a significant effect of grouping by immunoblot result.

Table 4-14. Effect of classification according to immunoblot result (positive, intermediate, or negative) on CSF *S. neurona* and IgG parameters ($\bar{x}$ ± sem).

WB result	*N*	*S. neurona* titer (EU/ml)	SN ratio*	IgG conc (mg/dl)	IgG ratio†	C	RQ
negative	25	17.7±6.4	0.37±0.04	15.4±1.7	0.49±0.05	0.89±0.09	0.00±0
intermediate	13	38.8±24.2	0.37±0.06	15.1±2.8	0.40±0.08	1.7±0.56	0.00±0
positive	24	2,351.1±992.9	0.65±0.07	15.5±2.3	0.46±0.07	2.08±0.3	19.38±3.96
All	62	925.4±406.1	0.48±0.04	15.4±1.3	0.46±0.04	1.52±0.18	7.50±1.94
P-value		<0.001	0.002	0.887	0.362	0.003	<0.001

* *S. neurona* titer CSF/*S. neurona* titer serum (×100)
† IgG concentration CSF/IgG concentration serum (×100)
C = CSF coefficient; *N* = Number; RQ = relative quantity CSF

Clinical Analysis

Cerebrospinal fluid (∀serum) was obtained from horses admitted to the Veterinary Medical Teaching Hospital at the University of Florida for evaluation of neurologic problems. These samples were sorted into three groups, depending on final diagnosis provided in the medical record. The groups were as follows: EPM, non-EPM (those horses diagnosed with some other neurological disease), and undiagnosed (those horses

in which a diagnosis was not established). Figures 4-11 and 4-12 show the Tukey's box plots of the coefficients (C) and RQ values from these three groups respectively. There was no significant effect of clinical group on any value except C (P = 0.005) and RQ (P <0.001). Post hoc pairwise analyses of C values did not reveal any significant difference between C values for EPM horses vs. other groups; however, there was a significant difference between the non-EPM and undiagnosed groups. For RQ values, significant difference was detected between EPM and non-EPM groups.

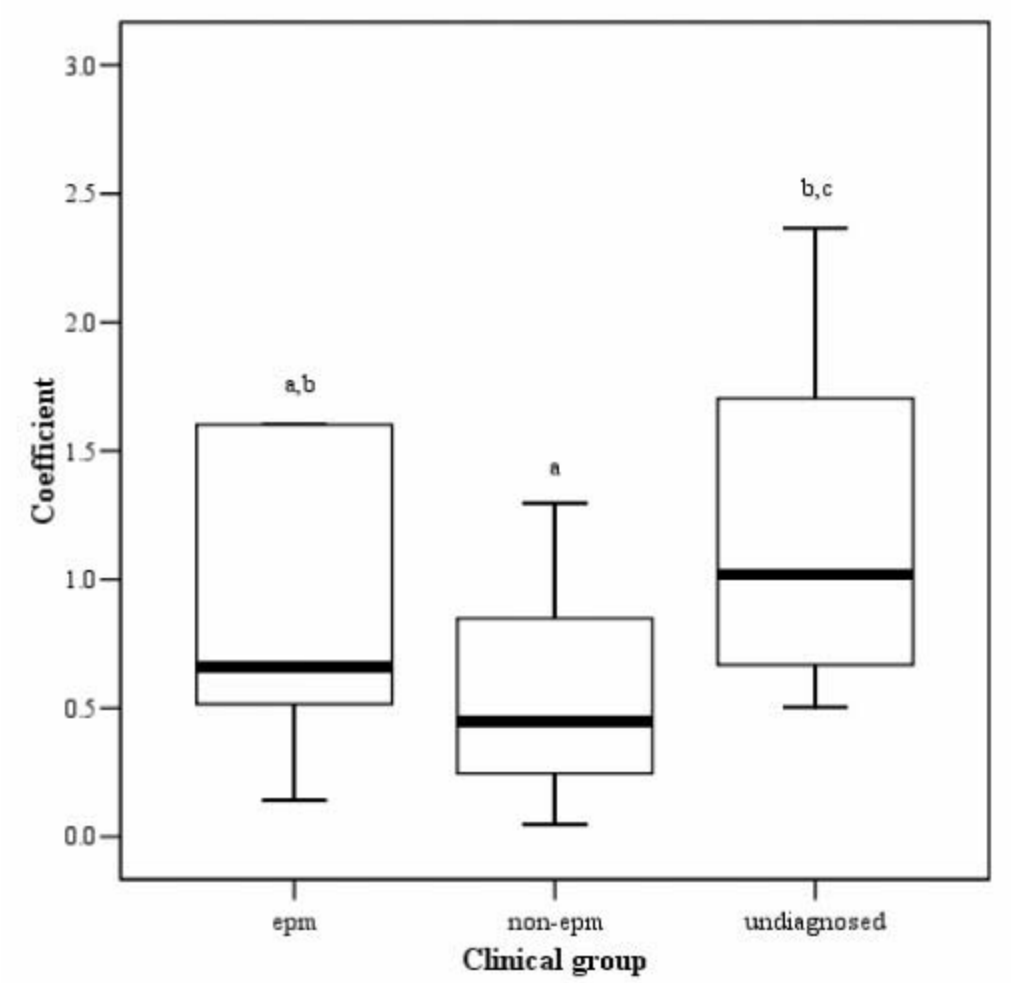


Figure 4-11. Tukey's box plot of coefficient value (C) from clinical sample groups. For each box, the central line is the median value, the margins of the box are the 1st and 3rd quartiles, and the error bars include these quartiles ∀1.5× the interquartile range. Outliers are not shown. Pairs of plots that do not share the same superscripted letter are significantly different.

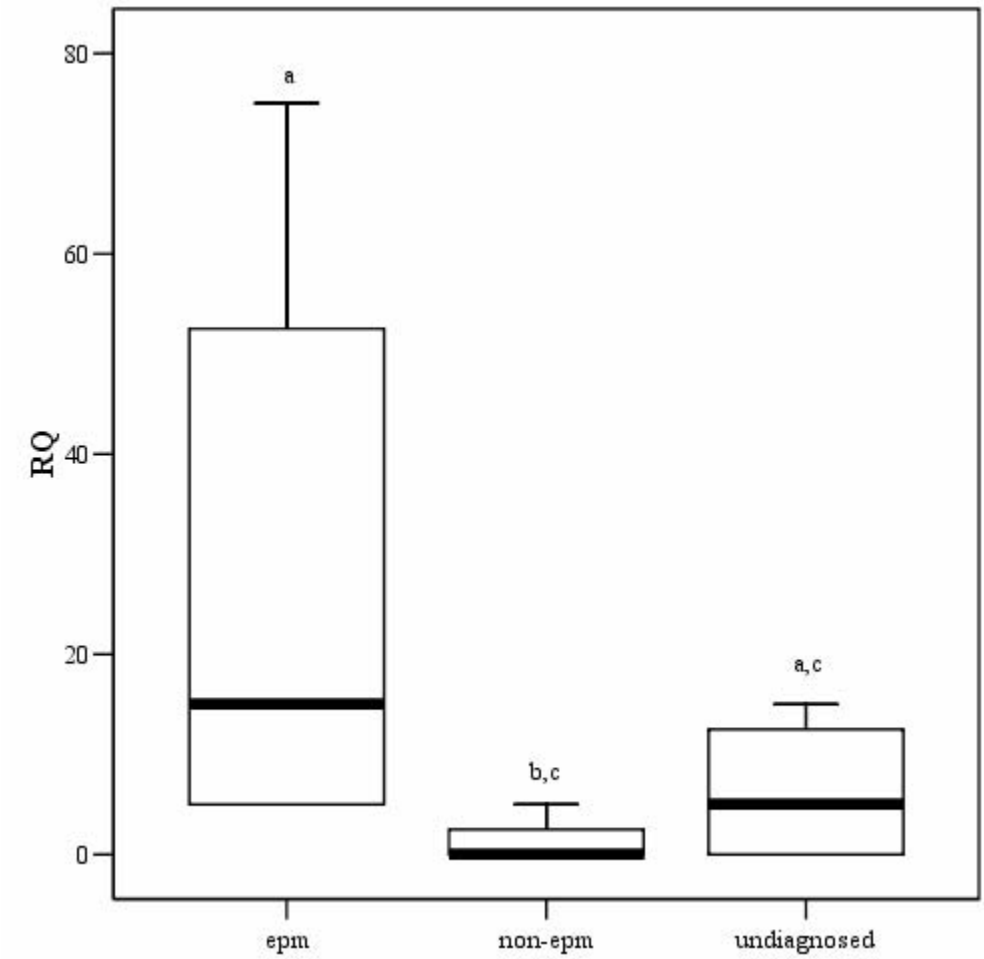


Figure 4-12. Tukey's box plot of RQ values from clinical sample groups. Pairs of values that do not share the same superscripted letter are significantly different.

Tables 4-15, 4-16, and 4-17 below show a combined analysis for all experimental horses before challenge, all untreated experimental horses after challenge, and the clinical groups already defined. There were significant effects of group on serum and CSF *S. neurona* titers, IgG ratio, C, and RQ. Post hoc pairwise analyses showed that the serum and CSF *S. neurona* titers in the pre-challenge experimental group were significantly different from those in all other groups. There was no consistent pattern of differences among groups for IgG ratio: both experimental groups were significantly different from non-EPM and undiagnosed clinical groups. The EPM group did not differ significantly from any other group. Pairwise analysis of C values among groups showed significant

difference between challenged and unchallenged experimental groups and between unchallenged, non-EPM, and undiagnosed groups. For RQ values, challenged and EPM groups were significantly different from unchallenged groups.

Table 4-15. Effect of group on *S. neurona* titer in experimental and clinical horses ($\bar{x}$ sem).

Group	*N*	SNs (EU/ml)	SNc (EU/ml)	SNr
sn-	31	4,465.1±1,141.0[a]	13.2±4.6[a]	0.39±0.04
Sn+	8	238,456.7±129,027.6[b]	1,444.5±855.8[b]	0.59±0.13
epm	9	131,998.8±35,866.4[b]	2,563.4±2,136.6[b]	2.60±1.92
non-epm	16	119,285.8±28,113.1[b]	297.2±82.0[b]	0.41±0.08
undiagnosed	12	80,491.3±17,372.6[b]	623.8±274.7[b]	0.74±0.23
P-value		<0.001	<0.001	0.055

sn- = pre-challenge experimental horses; sn+ = post-challenge experimental horses
SNs = serum *S. neurona* titer; SNc = CSF *S. neurona* titer; SNr - *S. neurona* ratio;
Pairs of values that do not share the same superscripted letter are significantly different.

Table 4-16: Effect of group on IgG concentrations in experimental and clinical horses ($\bar{x}$ ± sem).

Group*	*N*	IGs (mg/dl)	IGc (mg/dl)	IGr
sn-	31	3,506.2±175.3	15.0±1.7	0.44±0.05[a]
sn+	8	4,065.0±516.2	13.7±4.8	0.37±0.12[a]
epm	9	2,882.9±120.0	19.1±4.0	0.66±0.13[a,c]
non-epm	16	3,003.5±157.0	22.2±2.8	0.73±0.08[b,c]
undiagnosed	12	3,298.5±108.4	17.4±1.2	0.55±0.04[b,c]
P-value		0.094	0.086	0.003

sn- = pre-challenge experimental horses; sn+ = post-challenge experimental horses
IGs = IgG concentration in serum; IGc = IgG concentration in CSF; Igr = IgG ratio
Pairs of values that do not share the same superscripted letter are significantly different.

Table 4-17: Effect of group on C and RQ values in experimental and clinical horses ($\bar{x}$ ± sem).

Group*	*N*	C	RQ
sn-	31	1.44±0.28[a]	0.8±0.5[a]
sn+	8	2.57±0.74[b]	21.9±6.9[b]
epm	9	3.32±2.22[a,b,c]	28.1±10.3[b]
non-epm	16	0.55±0.09[c]	1.6±0.8[a,c]
undiagnosed	12	1.58±0.3[a,b]	7.8±2[b,c]
P-value		0.005	<0.001

sn- = pre-challenge experimental horses; sn+ = post-challenge experimental horses
C = coefficient; RQ = relative quantity CSF
Pairs of values that do not share the same superscripted letter are significantly different.

CHAPTER 5
DISCUSSION

Since EPM can be so devastating to the equine industry, there are active continuous efforts to study the disease. An important area needing additional research is the one addressed in this work – namely, diagnostic testing. The current standard-of-the-industry diagnostic test for EPM, the immunoblot, is presumed to be highly sensitive but has many false-positives; thus, the principal use of the immunoblot is as an aid in ruling out EPM. As a result of the work here, it is hoped that future diagnostic tests will be developed which may enable a more accurate diagnosis of the disease.

The project started with the development of methods for quantification of IgG in equine serum and CSF samples. It was originally thought a test kit was available for the quantification of IgG in equine serum samples. However, the kits that were available were found to be for individual subclasses of IgG (IgGa, IgGb, IgG(T) – Bethyl Laboratories, Inc, Montgomery, Texas) not total IgG. There was also no literature cited where such an assay had been used for equine total IgG in serum or CSF, so an assay was developed following general ELISA principles.

When serum samples were run in replicates using the developed assay, the inter- and intra-assay coefficients of variation were unacceptably high (both >30%). The reasons for this poor repeatability are not clear. There may have been interference of other proteins that are normally present in serum. Equine reference serum was used as the standard instead of purified IgG so that the samples and standard would contain the same proteins. This strategy did not seem to improve the results of the serum samples

run. Other means of quantifying the IgG in serum were explored and it was decided to use the radial immunodiffusion (RID) assay performed by Neogen, Inc. (Lexington, KY). The RID assay has been used for many years and has proven to be dependable in the determination of IgG in equine serum (Tizard, 2000)

When the RID was used to quantify IgG in serum samples from horses challenged with *S. neurona* sporocysts (or controls), results showed there was no significant effect of challenge within each group. However, the change in serum IgG concentration over time was significantly lower in horses given dexamethasone. Dexamethasone, an immunosuppressant, would be expected to suppress IgG production (Tizard, 2000)

By contrast with results from serum, when CSF samples were run in replicate on the developed ELISA the inter- and intra-assay coefficients of variation were much more acceptable (10.7% interassay and 9.1% intraassay) and yielded CSF IgG concentrations within the published normal limits of 3 - 10.5 mg/dl (Green et al., 1993). It is likely that the CSF ELISA was more dependable than the serum ELISA because CSF has less protein concentration and therefore less interference. There was no significant effect of time or treatment group on CSF IgG concentration in either experiment. The effect of time, as used here, is the difference between the after- and before-challenge values (i.e., the effect of challenge), seen within the groups.

The second objective of this study was to develop an ELISA to detect and quantify *S. neurona* IgG. Blood was collected from an experimental horse that had been immunized with *S. neurona* merozoites. Serum prepared from this blood was used as a positive control for all assays and referred to as “immune” serum. This immune serum was assigned a nominal value of 100,000 EU/ml. For this assay, it was hypothesized that

any *S. neurona*-reactive IgG that was produced locally in the CNS would be significant. We considered the possibility of another agent (i.e., non-*S. neurona*) being responsible for *S. neurona*-reactive IgG in the CSF as unlikely. *Neospora hughesi* is the only related pathogen shown to infect the equine CNS; however, infection with this organism is uncommon compared to *S. neurona*-associated EPM (Hamir et al., 1998; Marsh et al., 1998).

The results for the serum samples run on the *S. neurona* ELISA showed substantial and, in some groups, significant, increases in serum *S. neurona* titer in all *S. neurona* challenged groups. There is a suggestion that the immune response to *S. neurona* begins after there is replication (schizogony) in the invaded host (Franklin et al., 2003); thus, active infection may be required in order to stimulate a specific antibody response. This suggests that extraneural infection occurs in the horse, although *S. neurona* stages have only been found in CNS of immunocompetent horses. There was a significant effect of treatment group on *S. neurona* titer in both sets of experimental samples. The increase in titer over time was significantly greater in all challenged groups than it was in control groups. Seroconversion, as detected by immunoblot, has previously been seen in horses challenged with *S. neurona* sporocysts (Sofaly et al., 2002; Cutler et al., 2001).

It was expected that there would be an increase in *S. neurona* CSF titer in those experimental horses which were challenged with sporocysts. According to a previous report, mild to moderate clinical signs of neurologic disease were produced in challenged horses (Sofaly et al., 2002), indicating CNS infection with *S. neurona*. In these experiments, increased CSF titer was seen in all challenged groups over the period of the experiment. The effect of treatment group on this CSF titer increase was also significant.

The increase in titer was greater in all challenged groups than it was in the control groups.

These significant differences in *S. neurona* serum and CSF titers seen between groups for each experiment showed that infection with sporocysts did cause an increase in *S. neurona* titer. Higher mean titers were seen in experiment 2 where horses received a larger challenge dose of sporocysts. In experiment 1 horses were given only one dose of 5×10^5 sporocysts whereas in experiment 2 the horses were given a higher dose, receiving daily doses of 4×10^5 sporocysts for 7 days, for a total of 2.8×10^6 sporocysts. In serum, the *S. neurona* titer after challenge (no treatment) was 423,403.2 ± 233,556.7 EU/ml in experiment 2 compared to 53,510.1 ± 18,410.8 EU/ml in experiment 1. In CSF, the titer after challenge (no treatment) in the second experiment was 2542.4 ± 1606.4 EU/ml compared to 346.7 ± 184.2 EU/ml in experiment 1. This suggests that the antibody response to *S. neurona* infection may be dose-dependent and provides a context within which to evaluate titers seen in naturally infected horses. The ability to quantify *S. neurona* IgG in serum and CSF thus may be an advantage over the current immunoblot used for diagnosis.

The coefficient (C) also known as the antibody index (Reiber & Lange, 1991), has been used to differentiate antibodies produced locally from those produced systemically (Lappin et al., 1996b). Antibody indexes are used to detect specific antibody in CSF (Reiber et al., 2001). This type of antibody index has been used in various diseases including Lyme borreliosis in humans (caused by *Borrelia burgdorferi*) (Hammers-Berggren et al., 1993), and sleeping sickness in humans (caused by *Trypanosoma brucei gambiense*) (Bisser et al., 2002), multiple sclerosis in humans (Felgenhauer & Reiber,

1992) among others (Reiber & Peter, 2001). On theoretical grounds, in the absence of CNS disease, the mean normal value of C (or AI) should be 1.0 (Felgenhauer & Reiber, 1992). Based on a study looking at antibody indexes in multiple sclerosis of humans, a range of 0.7-1.3 was established as the reference range for normal human beings; anything above this indicates CNS disease (Felgenhauer & Reiber, 1992; Reiber et al., 2001). In our work here, the C values rose slightly after challenge (from mean value of 1.44), although this increase was not significant. On the basis of the results in the very small population, of *S. neurona* naïve horses sampled in this study, the upper 95% confidence limit for uninfected horses is 2.27. This value is artificially high because the high background encountered with certain CSF samples interfered with the sensitivity of the *S. neurona* assay (see Results chapter). The reason for this background in occasional samples was not discerned. It will be important to eliminate this occasional problem in order to establish a true reference range for C.

Although C-values were increased or unchanged after challenge in all groups, this change was not significant. There is a variety of reasons why significant increase in C was not found in *S. neurona*-challenged horses. To begin with, the formula itself contains four different variables, each with individual measurement variability. This variability is increased multiplicatively through the operations of the formula. This can cause a large range in normal values and obscure the potential significance of small changes.

Importantly, the rise in CSF *S. neurona* titer without associated rise in C that was observed in this study may suggest that *S. neurona* IgG found in CSF was all of *systemic* origin – i.e. *that there was no CNS infection*. In support of this notion, *S. neurona*

organisms have never been identified in the CNS of experimentally infected horses and were not found in horses that were the subject of the present studies. Furthermore, significant abnormal neurologic signs were not seen in these horses after 2 different *S. neurona* challenge protocols.

There could have been potential errors with the assays that may have played a role in C values obtained. High background seen in some CSF samples may have lead to artificially high C values. General laboratory errors may have caused a variation in the individual concentrations and titers obtained on the ELISA. The antibodies were stored following manufacturer's recommendations but if there was a breakdown results may have been affected. For instance, if the HRP conjugated IgG, broke down and the HRP was no longer attached to all the IgG molecules then the reading would have been less.

Another problem with the assays could have stemmed from the samples themselves. Crowther (1995) implies that serum needs to be mixed upon thawing to ensure homogeneity in the sample, as protein tends to settle at the bottom of the tube when freezing. This may have been an issue in our assays since our samples were all stored at -80C and thawed for each assay, if they were not mixed completely to allow homogeneity. If our samples were not mixed appropriately, the IgG in the samples may not have been evenly distributed throughout the sample.

The next step in the analysis involved classifying our data according to immunoblot result. All samples from both experiments were sorted by immunoblot result according to 2-level and 3-level immunoblot classification systems.

According to the analysis of the 2-level immunoblot classification, the mean values for serum and CSF *S. neurona* titer, SN ratio, C value and RQ were significantly higher

for the samples classified as immunoblot-positive than they were for those classified as negative. The difference in titers is substantial in both serum and CSF, with over a 40 times increase in serum and a 100 times increase in CSF. The increase in C value shows that use of the coefficient may be comparable to diagnosis based on immunoblot test results. Relative quantity values would be expected to rise in those horses that are positive, as an increase in the RQ value is seen in those horses diagnosed positive by immunoblot for EPM.

In the analysis of the 3-level immunoblot classification, significant effect of this classification was found for *S. neurona* serum and CSF titers, *S. neurona* ratio, C value and RQ. Pairwise analyses could have been performed but were not and therefore it could not be determined how the groups compare to each other (i.e., which groups are different from each other). In this classification only 2 horses were classified as negative which may have resulted in unreliable results (i.e., if one was an outlier).

For analysis of clinical data, samples were grouped according to broad categories of diagnosis as listed in the official medical record. The *EPM group* was comprised of horses that were diagnosed with EPM from clinical signs, immunoblot test results; and, in rare cases necropsy results; the *non-EPM group* was those horses that were definitively diagnosed with another neurological disease on the basis of clinical findings and other test results; and the undiagnosed group was those horses with suspected neurologic disease for which a diagnosis was never reached. A major issue of these groups is the accuracy of diagnosis. The gold standard for EPM diagnosis is necropsy and necropsy results were available for only a few of the cases used for this series.

The clinical sample data showed that there was a significant effect of diagnosis classification only on the C value and RQ value. C values were only significantly different between the non-EPM and the undiagnosed groups. These results, based on very small numbers, do not appear to support the use of C-values to distinguish horses with EPM from those with other neurologic disease. As stated above, however, the diagnoses in these cases were usually clinical and may not have always been accurate. The significant difference for the RQ was between EPM and non-EPM groups. This difference was expected as RQ values, which reflect amount of *S. neurona* antibody in CSF (Cutler et al., 2001), should be higher in those horses with EPM than those without EPM (MacKay et al., 2000). The small sample, which contained only neurologically abnormal horses, is not representative of an entire population and therefore future studies with larger sample sizes should be considered. These studies could include groups such as clinically normal horses, neurologic horses with diseases other than EPM, horses diagnosed with EPM, *S. neurona* naïve horses, and clinically normal horses naturally exposed to *S. neurona*. Higher numbers and the variety of groups would allow further exploration of clinical application.

When the clinical data were combined with those for experimental horses, significant effects of group (i.e., experimental negative controls, *S. neurona*-challenged, EPM, non-EPM, undiagnosed) on the *S. neurona* serum and CSF titers, IgG ratio, C value, and RQ value were observed. With respect to *S. neurona* titers, we found that the results for challenged horses were higher than those for any other group. Since the results were based on experimentally challenged horses, we cannot determine how the results would compare with horses naturally infected with *S. neurona*. When reviewing

the *S. neurona* serum titers, it was noticed that the mean serum value of 110,592.0 EU/ml for all clinical horses was somewhat comparable to 238,456.7 EU/ml for challenged horses, but much higher than 4465.1 EU/ml for pre-challenged, *S. neurona* naïve horses. In CSF, the mean titer value of 1161.5 EU/ml for all clinical horses was similar to the 1444.5 EU/ml value for the challenged horses, but much higher than the 13.2 EU/ml titer in the pre-challenged, *S. neurona* naïve horses. Because we only have one time point after infection, we do not know if the results would have been higher earlier in infection. The IgG ratio results varied across groups but not in any discernibly consistent way. The C value differed significantly between the pre-challenged and post-challenged groups and between the non-EPM and undiagnosed groups. The former result provides evidence that when horses are infected with *S. neurona*, C values may rise, although similar analyses within experiments did not reveal significant increase (see above). Similarly, RQ values differed significantly before and after challenge. The other significant difference for RQ was between the EPM group and the pre-challenged group. Both of these differences for RQ were expected since RQ should go up when there is a *S. neurona* infection of the CNS (Cutler et al., 2001).

From this study we have determined how to quantify the antibody response to *S. neurona* infection. The data presented in this study were based primarily on experimental challenges and yielded encouraging results; however, nothing conclusive can be determined about natural infections. Data for experimental challenge studies can be interpreted as suggesting that active infection of *S. neurona* may not occur in the CNS even though *S. neurona*-reactive IgG is found in the CSF of experimentally-infected horses. This preliminary finding, if supported, challenges the validity of this sporocyst

infection technique as a model for EPM. Future studies involving a larger clinical population could establish a normal range for C and provide application to naturally infected horses.

The coefficient, C, as defined in this study, shows some promise as an aid to diagnosis of EPM; however, insufficient evidence was developed to support its use without considerable further study. Whether or not an actual upper cutoff for C can be established that distinguishes CNS-origin *S. neurona* antibody from that produced systemically has not yet been determined. It is hoped that the data from challenge studies reported here will provide a strong foundation for future work. The number of clinical cases would have to increase to have a better understanding of how the coefficient, C, could impact the diagnosis of naturally infected horses.

CHAPTER 6
CONCLUSION

During this study we developed an ELISA for the quantification of IgG in equine CSF. We also developed a method to quantify the antibody response to *S. neurona* infection. The data presented in this study were based primarily on experimental challenges and yielded encouraging results; however, nothing conclusive can be determined about natural infections.

The coefficient, C, as defined in this study, shows some promise as an aid to diagnosis of EPM; however, insufficient evidence was developed to support its use without considerable further study. Future studies involving a larger clinical population could establish a normal range for C and provide application to naturally infected horses. Whether or not an actual upper cutoff for C can be established that distinguishes CNS-origin *S. neurona* antibody from that produced systemically has not yet been determined. It is hoped that the data from challenge studies reported here will provide a strong foundation for future work. Through the work presented here it is hoped that advancement can be made in the area of EPM diagnosis.

LIST OF REFERENCES

Bisser, S., Lejon, V., Preux, P.M., Bouteille, B., Stanghellini, A., Jauberteau, M.O., Buscher, P., Dujas, M. 2002. Blood-cerebrospinal fluid barrier and interthecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness. J. Neuro Sciences 193, 127-135.

Boy, M.G., Galligan, D.T., Divers, T.J., 1990. Protozoal encephalomyelitis in horses: 82 cases (1972-1986). J. Am. Vet. Med. Assoc. 196, 632-634.

Cheadle, M.A., Lindsay, D.S., Rowe, S., Dykstra, C.C., Williams, M.A., Spencer, J.A., Toivio-Kinnucan, M.A., Lenz, S.D., Newton, J.C., Rolsma, M.D., Blagburn, B.L. 1999. Prevalence of antibodies to *Neospora caninum* in dogs. Int. J. Parasitol. 29, 1537-1543.

Cheadle, M.A., Tanhauser, S.M., Dame, J.B., Sellon, D.C., Hines, M., Ginn, P.E., MacKay, R.J., Greiner, E.C, 2001a. The nine-banded armadillo (*Dasypus novemcinctus*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31,330-335.

Cheadle, M.A., Yowell, C.A., Sellon, D.C., Hines, M., Ginn, P.E., Marsh, A.E., Dame, J.B., Greiner, E.C., 2001b. The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31, 843-849.

Cohen, N.D., MacKay, R.J., 1997. Interpreting immunoblot testing of cerebrospinal fluid for equine protozoal myeloencephalitis. The Compendium October, 1176-1181.

Crowther, J.R. 1995. ELISA: theory and practice. Humana Press, Inc. Totowa, New Jersey.

Cserr, H.F., Knopf, P.M., 1992. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. Immunology Today 13, 507-512.

Cusick, P.K., Sells, D.M., Hamilton, D.P., Hardenbrook, H.J., 1974. Toxoplasmosis in two horses. J. Am. Vet. Med. Assoc. 164, 77-80.

Cutler, T.J., 1999. The host infectivity spectrum of *Sarcocystis neurona* and *Sarcocystis falcatula*. M.S. Thesis University of Florida, Gainesville.

Cutler, T.J., MacKay, R.J., Ginn, P.E., Tanhauser, S.M., LeRay, E.V., Dame, J.B., Greiner, E.C. 2001. Immunoconversion against *Sarcocystis neurona* in normal and dexamethasone-treated horses challenged with *S. neurona* sporocysts. Vet. Parasitol. 95. 197-210.

Daft, B., Ardans, A., Barr, B., Hird, D., Hietala, S., 1997. A comparison of antemortem and postmortem testing for *Sarcocystis neurona* with postmortem examination of neurologically normal and abnormal California horses. In: Proceedings of the Abstracts of the 40th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, Lousiville, KY, p. 86.

Daft, B.M., Barr, B.C., Gardner, I.A., Reed, D., Bell, W., Peyser, K.G., Ardans, A., Kinde, H., Morrow, J.K., 2002. Sensitivity and specificity of western blot testing of cerebrospinal fluid and serum for diagnosis of equine protozoal myeloencephalitis in horses with and without neurologic abnormalities. J. Am. Vet. Med. Assoc. 221, 1007-1013.

Davis, S.W., Daft, B.M., Dubey, J.P., 1991a. *Sarcocystis neurona* cultured in vitro from a horse with equine protozoal myelitis. Equine. Vet. J. 23, 315-317.

Davis, S.W., Speer, C.A., Dubey, J.P., 1991b. In vitro cultivation of *Sarcocystis neurona* from the spinal cord of a horse with equine protozoal myelitis. J. Parastiol. 77, 789-792.

Duarte, P.C., Daft, B.M., Conrad, P.A., Packham, A.E., Gardner, I.A., 2003. Comparison of a serum indirect fluorescent antibody test with two western blot tests for the diagnosis of equine protozoal myeloencephalitis. J. Vet. Diagn. Invest. 15, 8-13.

Duarte, P.C., Daft, B.M., Conrad, P.A., Packham, A.E., Saville, W.J., MacKay, R.J., Ng, T., Gardner, I.A., 2004. Evaluations and comparisons of an indirect fluorescent antibody test for detection of antibodies to *Sarcocystis neurona* using serum and cerebrospinal fluid of naturally-, experimentally-infected and vaccinated horses. J. Parasitol, in press.

Dubey, J.P., 1976. A Review of *Sarcocystis* of Domestic Animals and Other Coccidia of Cats and Dogs. J. Am. Vet. Med. Assoc. 169, 1061-1078.

Dubey, J.P., Davis, S.E., Speer, C.A., Bowman, D.D., de Lahunta, A., Granstrom, D.E., Topper, M.J., Hamir, A.N., Cummings, J.F., Suter, M.M.., 1991. *Sarcocystis neurona* N. Sp. (Protozoa: Apicomplexa), The etiologic agent of equine protozoal myeloencephalitis. J. Parasitol. 77, 212-218.

Dubey, J.P., Liddel, S., Mattson, D., Speer, C.A., Howe, D.K., Jenkins, M.C. 2001a. Characterization of the Oregon isolate of *Neospora hughesi* from a horse. J. Parasitol. 87, 345-353.

Dubey, J.P., Lindsay, D.S., Saville, W.J.A., Reed, S.M., Granstrom, D.E., Speer, C.A., 2001b. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Vet. Parasit. 95, 89-131.

Dubey, J.P., Mattson, D.E., Speer, C.A., Baker, R.J., Mulrooney, D.M., Tornquist, S.J., Hamir, A.N., Gerros, T.C., 1999. Characterization of *Sarcocystis neurona* isolate (SN6) from a naturally infected horse from Oregon. J. Eukaryt. Microbiol. 46, 500-506.

Dubey, J.P., Mattson, D.E., Speer, C.A., Hamir, A.N., Lindsay, D.S., Rosenthal, B.M., Kwok, O.C.H., Baker, R.J., Mulrooney, D.M., Tornquist, S.J., Gerros, T.C., 2001c. Characteristics of a recent isolate of *Sarcocystis neurona* (SN7) from a horse and loss of pathogenicity of isolates SN6 and SN7 by passages in cell culture. Vet. Parasitol. 95, 155-166.

Dubey, J.P., Saville, J.A., Lindsay, D.S., Stich, R.W., Stanek, J.F., Speer, C.A., Rosenthal, B.M., Njoku, C.J., Kwok, O.C.H., Shen, S.K., Reed, S.M. 2000. Completion of the life cycle of *Sarcocystis neurona*. J. Parasitol. 86, 1276-1280.

Dubey, J.P., Saville, J.A., Stanek, J.F., Lindsay, D.S., Rosenthal, B.M., Oglesbee, M.J., Rosypal, A.C., Njoku, C.J., Stich, R.W., Kwok, O.C.H., Shen, S.K., Hamir, A.N., Reed, S.M. 2001d. *Sarcocystis neurona* infection in raccoons (*Procyon lotor*): evidence for natural infection with sarcocystis, transmission fo infection to opossums (*Didepphis virginiana*), and experimental induction of neurologic disease in raccoons. Vet. Parasit. 100, 117-129.

Dubey, J.P., Speer, C.A., Fayer, R., 1999. General biology. In Sarcocystosis of Animals and man. Boca Raton, CRC Press, 1-91.

Felgenhauer, K., Reiber, H. 1992. The diagnostic significance of antibody specificity indices in multiple sclerosis and herpes virus induced disaseas of the nervous system. Clin. Invest. 70, 28-37.

Fenger, C.K., Granstrom, D.E., Gajadhar, A.A., Williams, N.M., McGrillis, S.A., Stamper, S., Langemeier, J.L., Dubey, J.P., 1997. Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis* sp. Sporocysts from the opossum (*Didelphis virginiana*). Vet. Parasitol. 68, 199-213.

Fenger, C.K., Granstrom, D.E., Langemeier, J.L., Stamper, S., Donahue, J.M., Patterson, J.S., Gajadhar, A.A., Marteniuk, J.V., Xiaomin, Z., Dubey, J.P., 1995. Identification of opossums (*Didelphis virginiana*) as the putative definitive host of *Sarcocystis neurona*. J. Parasitol. 81, 916-919.

Franklin, R.P., MacKay, R.J., Gillis, K.D., Tanhauser, S.M., Ginn, P.E., Kennedy, T.J. 2003. Effect of a single dose of ponazuril on neural infection and clinical disease in *Sarcocystis neurona*-challenged interferon-gamma knockout mice. Vet. Parasitol. 114, 123-130.

Gillis, K.D., 2003. Naturally occurring *Sarcocystis* infection in domestic cats (*Felis catus*). M.S. Thesis University of Florida, Gainesville.

Gillis, K.D., MacKay, R.J., Yowell, C.A., Levy, J.K., Greiner, E.C., Dame, J.B., Cheadle, M.A., Hernandez, J., Massey, E.T. 2003. Naturally occurring *Sarcocystis* infection in domestic cats (*Felis catus*). Int. J. Parasitol. 33, 877-883.

Granstrom, D.E., 1997. Equine protozoal myeloencephalitis: Parasite biology, experimental disease, and laboratory diagnosis. In Proceedings of the International Equine Neurology Conference, Ithaca, New York , 4-6.

Granstrom, D.E., Alvarez, O., Dubey, J.P., Comer, P.F., Williams, N.M., 1992. Equine protozoal myelitis in Panamanian horses and isolation of *Sarcocystis neurona*. J. Am. Vet. Med. Assoc. 78, 909-912.

Granstrom, D.E., Dubey, J.P., Davis, S.W., Fayer, R., Fox, J.C., Poonacha, K.B., Giles, R.C., Comer, P.F., 1993. Equine protozoal myeloencephalitis: antigen analysis of cultured *Sarcocystis neurona* merozoites. J. Vet. Diagn. Invest. 5, 88-90.

Granstrom, D.E., MacPherson, J.M., Gajadhar, A.A., Dubey, J.P., Tramontin, R., Stamper, S., 1994. Differentiation of *Sarcocystis neurona* from eight related coccidian by random amplified polymorphic DNA assay. Mol. Cell. Probes. 8, 353-356.

Green, E.M., Gheorghe, C.M., Kroll, R.A. 1993. Equine cerebrospinal fluid: analysis. Compendium 15, 288-302.

Hamir, A.N., Moser, G., Galligan, D.T., Davis, S.W., Granstrom, D.E., Dubey, J.P., 1993. Immunohistochemical study to demonstrate *Sarcocystis neurona* in equine protozoal myeloencephalitis. J. Vet. Diagn. Invest. 5, 418-422.

Hamir, A.N., Tornquist, S.J., Gerros, T.C., Topper, M.J., Dubey, J.P. 1998. *Neospora caninum*-associated equine protozoal myeloencephalitis. Vet. Parasitol. 79, 269-274.

Hammers-Berggren, S., Hansen, K., Lebech, A-M., Karlsson, M. 1993. *Borrelia burgdorferi*-specific intrathecal antibody production in neuroborrleiosis: a follow-up study. Neurology 43, 169-175.

Lapointe, J.M., Duignan, P.J., Marsh, A.E., Gulland, F.M., Barr, B.C., Naydan, D.K., King, D.P., Farman, C.A., Huntingdon, K.A.B., Lowenstine, L.J., 1998. Meningoencephalitis due to a *Sarcocystis neurona*-like protozoan in Pacific harbor seals (*Phoca vitulina richardsi*). J. Parasitol. 84, 1184-89.

Lappin, M.R., Burney, D.P., Dow, S.W., Potter, T.A., 1996b. Polymerase chain reaction for the detection of *Toxoplasma gondii* in aqueous humor of cats. Am. J. Vet. Res. 57, 1589-1593.

Lappin, M.R., Chavkin, M.J., Munana, K.R., Cooper, C.M. 1996a. Feline ocular and cerebrospinal fluid *Toxoplasma gondii*-specific humoral immune responses following specific and nonspecific immune stimulation. Vet. Imm. Immunopath. 55, 23-31.

Liang, F.T., Granstrom, D.E., Zhao, X.M., Timoney, J.F., 1998. Evidence that surface proteins Sn14 and Sn16 of *Sarcocystis neurona* merozoites are involved in infection and immunity. Infect. Immun. 66, 1981-1983.

Lindsay, D.S., Thomas, N.J., Dubey, J.P., 2000. Biological characterization of *Sarcocystis neurona* from a Southern sea otter (*Enhydra lutris nereis*). Int. J. Parasitol. 30, 617-624.

MacKay, R.J., 1997a. Equine protozoal myeloencephalitis. In: Veterinary Clinics of North America: Equine Practice 13, 79-96.

MacKay, R.J., 1997b. Serum antibodies to *Sarcocystis neurona* – half the horses in the United States have them! J. Am. Vet. Med. Assoc. 210, 482-483.

MacKay, R.J., Granstrom, D.E., Saville, W.J., Reed, S.M., 2000. Equine protozoal myeloencephalitis. Vet. Clin. N. Am.: Eq. Pract. 16, 405-425.

Mansfield, L.S., Schott II, H.C., Murphy, A.J., Rossano, M.G., Tanhauser, S.M., Patterson, J.S., Nelson, K., Ewart, S.L., Marteniuk, J.V., Bowman, D.D., Kaneene, J.B., 2001. Comparison of *Sarcocystis neurona* isolates derived from horse neural tissue. Vet. Parasitol. 95, 167-178.

Marsh, A.E., Barr, B.C., Madigan, J., Lakritz, J., Conrad, P.A., 1996a. Sequence analysis and polymerase chain reaction amplification of small subunit ribosomal DNA from *Sarcocystis neurona*. Am. J. Vet. Res. 57, 975-981.

Marsh, A.E., Barr, B.C., Tell, L., Koski, M., Greiner, E., Dame, J., Conrad, P.A., 1997. In vitro cultivation and experimental inoculation of *Sarcocystis falcatula* and *Sarcocystis neurona* merozoites into budgerigars (*Melopsittacus undulates*). J. Parasitol. 83, 1189-1192.

Marsh, A.E., Bradd, B.C., Madigan, J.M., Lakritz, J., Nordhausen, M.A., Conrad, P.A., 1996b. Neosporosis as a cause of equine protozoal myeloencephalitis. J. Am. Vet. Med. Assoc. 209, 1907-1913.

Marsh, A.E., Bradd, B.C., Packham, A.E., Conrad, P.A. 1998. Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). J. Parasitol. 84, 983-991.

Marsh, A.E., Howe, D.K., Wang, G., Barr, B.C., Cannon, N., Conrad, P.A. 1999. Differentiaion of *Neospora hughesi* from *Neospora caninum* based on their immunodominant surface antigen, SAG1 and SRS2. Int. J. Parasitol. 29, 1575-1582.

Mayhew, I.G., de Lahunta, A., Whitlock, R.H., Pollock, R.V.H., 1976. Equine protozoal myeloencepahlitis. In Proceedings of 22nd Annual Convention of the American Association of Equine Practioners, Dallas, TX, Nov.-Dec, 107-114.

National Animal Health Monitoring System Equine '98: Equine protozoal myeloencephalitis in the US.1998. Fort Collins, Veterinary Services, United States Department of Agriculture Animal and Plant Health Inspection Service.

Reiber, H., Lange, P. 1991. Quantification of virus-specific antibodies in cerebrospinal fluid and serum: sensitive and specific detection of antibody synthesis in brain. Clin. Chem. 37, 1153-1160.

Reiber, H., Peter, J.B. 2001. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. J. Neuro. Sciences. 184, 101-122.

Rooney, J.R., Prickett, M.E., Delaney, F.M., Crowe, M.W., 1969. Focal Myelitis-Encephalitis in Horses. Cornell Vet. 60, 494-501.

Rossano, M.G., Mansfield, L.S., Kaneene, J.B., Murphy, A.J., Brown, C.M., Schott II, H.C., Fox, J.C., 2000. Improvement of western blot test specificity for detecting equine serum antibodies to *Sarcocystis neurona*. J. Vet. Diagn. Invest. 12, 28-32.

Saville, W.J., Morley, P.S., Reed, S.M., Granstrom, D.E., Kohn, C.W., Hinchcliff, K.W., Wittum, T.E., 2000a. Evaluation of risk factors associated with clinical improvement and survival of horses with equine protozoal myeloencephalitis. J. Am. Vet. Med. Assoc. 217, 1181-1185.

Saville, W.J., Reed, S.M., Granstrom, D.E., Hinchcliff, K.W., Kohn, C.W., Wittum, T.E., Stamper, S., 1997. Seroprevalence of antibodies to *Sarcocystis neurona* in horses residing in Ohio. J. Am. Vet. Med. Assoc. 210, 519-524.

Saville, W.J., Reed, S.M., Morley, P.S., Granstrom, D.E., Kohn, C.W., Hinchcliff, K.W., Wittum, T.E., 2000b. Analysis of risk factors for the development of equine protozoal myeloencephalitis in horses. J. Am. Vet. Med. Assoc. 217, 1174-1180.

Sofaly, C.D., Reed, S.M., Gordon, J.C., Dubey, J.P., Oglesbee, M.J., Njoku, C.J., Grover, D.L., Saville, W.J.A. 2002. Experimental induction of equine protozoal myeloencephalitis (EPM) in the horse: effect of *Sarcocystis neurona* sporocysts inoculation does on the development of clinical neurologic disease. J. Parasitol. 88, 1164-1170.

Tanhauser, S.M., Cheadle, M.A., Massey, T.E., Mayer, B.A., Schroedter, D.E., Dame, J.B., Greiner, E.C., MacKay, R.J., 2001. The nine-banded armadillo (*Dasypus novemcinctus*) is naturally infected with *Sarcocystis neurona*. Int. J. Parasitol. 31, 325-329.

Tizard, I. 2000. Veterinary Immunology. W.B. Saunders, Philadelphia, Pennsylvania. p. 216-217 & 426-428.

Turray, H.O., Barr, B.C., Caldwell, A., Branson, K.R., Cockrell, M.K.R., Marsh, A.E. 2002. *Sarcocystis neurona* reacting antibodies in Missouri feral domestic cats (*Felis domesticus*) and their role as an intermediate host. Parasitol. Res. 88, 38-43.

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service Report, 1995. June 1996.

United States Department of Agriculture (USDA), NAHMS Equine '98 Report. May 1997.

BIOGRAPHICAL SKETCH

Katherine Anne Heskett (maiden name Jensen) was born on February 24th, 1977, in Bridgeport, Connecticut, to parents Patricia Jensen and Edward Jensen. She grew up in Bridgeport and attended public school. She attended high school at Trumbull High in Trumbull, Connecticut. There was enrolled in the vocational agriculture program. She has always had a fondness for animals. In 1995, upon graduation from high school, Katherine moved to Bradenton, Florida, and began her undergraduate work at the University of South Florida in Tampa and began work on a major in biology. In 1998, she transferred to the University of Florida in Gainesville and graduated in August of 1999 with a Bachelor of Science degree in animal biology. Katherine took post-baccalaureate classes for a semester and then enrolled in graduate school at the College of Veterinary Medicine at the University of Florida in the Department of Large Animal Clinical Sciences. After receipt of her master's degree in veterinary medical sciences, Katherine plans to pursue a career as a biologist.