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EQUINE PROTOZOAL MYELOENCEPHALITIS: INVESTIGATION OF GENETIC SUSCEPTIBILITY AND ASSESSMENT OF AN EQUINE INFECTION METHOD

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THESIS

A thesis submitted in partial fulfillment of the
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By

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Lexington, KY

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2013

ABSTRACT OF THESIS

EQUINE PROTOZOAL MYELOENCEPHALITIS: INVESTIGATION OF GENETIC SUSCEPTIBILITY AND ASSESSMENT OF AN EQUINE INFECTION METHOD

Equine protozoal myeloencephalitis (EPM) is a progressive neurological disease of horses caused by *Sarcocystis neurona*. Two projects were conducted to identify factors involved in the development of EPM. The first study explored a possible genetic susceptibility to EPM by attempting a genome-wide association study (GWAS) on formalin-fixed, paraffin-embedded (FFPE) tissue from 24 definitively-positive EPM horses. DNA extracted from tissues older than 14 months was inadequate for SNP analysis on the Illumina Equine SNP50 BeadChip probably due to degradation and formalin cross-linking. Results were inconclusive as analysis was not possible with the small sample set. The second study evaluated an artificial infection method in creating a reliable equine EPM model. Five horses were injected intravenously at 4 time points with autologous blood incubated with 1,000,000 *S. neurona* merozoites. Challenged horses progressively developed mild to moderate clinical signs and had detectable *S. neurona* serum antibodies on day 42 post challenge. Horses appeared to have produced a Th1 immune response and cleared the infection by the conclusion of the study on day 89. No histopathological evidence of *S. neurona* infection was found within central nervous system tissue. This artificial infection method was not effective in replicating the severe clinical EPM seen in natural infections.

KEYWORDS: Equine protozoal myeloencephalitis, *Sarcocystis neurona*, Genome-wide association study, Experimental infection, Immune response

Breanna Marie Gaubatz
April 11, 2013

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CHAPTER ONE

Literature Review

1.1. Introduction

Equine protozoal myeloencephalitis (EPM) is an equine neurological disease. The disease was first described in 1970 (Rooney et al., 1970), and protozoa were identified within lesions from EPM horses in 1974 (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974). The apicomplexan parasite *Sarcocystis neurona* is the etiologic agent of EPM (Dubey et al., 1991). Following the range of the definitive host, the opossum (*Didelphis virginiana*, *Didelphis albiventris*) (Dubey and Lindsay, 1998; Dubey et al., 2001a), EPM is only found in the western hemisphere. Horses are aberrant hosts, and become infected by ingesting feed or water that is contaminated with opossum feces (Dubey and Lindsay, 1998). The disease displays clinical signs ranging from mild lameness to recumbency, and even death. Classic EPM clinical signs are asymmetrical ataxia with focal muscle atrophy (Dubey et al., 2001b).

Currently, the most accurate antemortem diagnosis of EPM is based on neurological signs consistent with EPM and a positive ELISA test for *S. neurona* antibodies in the serum and CSF (Furr et al., 2002; Yeargan and Howe, 2011). A definitive diagnosis can only be made during a postmortem examination when *S. neurona* is located histologically within the CNS (Furr et al., 2002). Over half of the horses in the United States are seropositive for *S. neurona* (MacKay, 1997b), while only 0.5-1% actually develop the disease (Dubey et al., 2001b).

1.2. History

A neurologic disease, referred to as “focal myelitis-encephalitis,” was first described in 52 Kentucky and Pennsylvania horses in 1970 (Rooney et al., 1970). Protozoa were first witnessed within central nervous system (CNS) lesions of affected horses in 1974 (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974). Initially, the disease-causing protozoa was misidentified as *Toxoplasma gondii* (Cusick et al., 1974), but subsequent evidence determined it was a *Sarcocystis* spp. due to its morphology and antigenic properties (Simpson and Mayhew, 1980). In 1976, Mayhew et al. were the first to name the disease, equine protozoal myeloencephalitis (EPM) (Mayhew et al., 1976). Studies of previous EPM cases indicated that a single parasitic agent was located within the lesions (Dubey et al., 1991). In 1991, an organism was isolated from a naturally infected horse with EPM (Davis et al., 1991b), given the name *Sarcocystis neurona* based on the location of the parasite within the horse and identified as the causative agent of EPM (Dubey et al., 1991). An immunohistochemical study of past EPM cases determined that most cases were caused by *S. neurona* (Hamir et al., 1993). There have been some reports of rare EPM cases caused by *Neospora* spp (Hamir et al., 1998; Marsh et al., 1996). This organism was isolated from a diseased horse in 1998 and established as a new species, *Neospora hughesi* (Marsh et al., 1998).

1.3. Phylogeny and Life Cycle of *Sarcocystis neurona*

Sarcocystis neurona is a parasite belonging to the phylum Apicomplexa and family Sarcocystidae (Beck et al., 2009), along with other cyst-forming coccidians, such as *T.*

gondii and *Neospora caninum* (Fenger et al., 1994) (Figure 1.1). Apicomplexan parasites are responsible for several major human and animal diseases (i.e., cryptosporidiosis, malaria, coccidiosis, toxoplasmosis, neosporosis) (Beck et al., 2009). They are relatively host specific, unicellular eukaryotes that contain an apical complex, consisting of unique cellular organelles that aid the parasite in penetrating host cells (Levine, 1970).

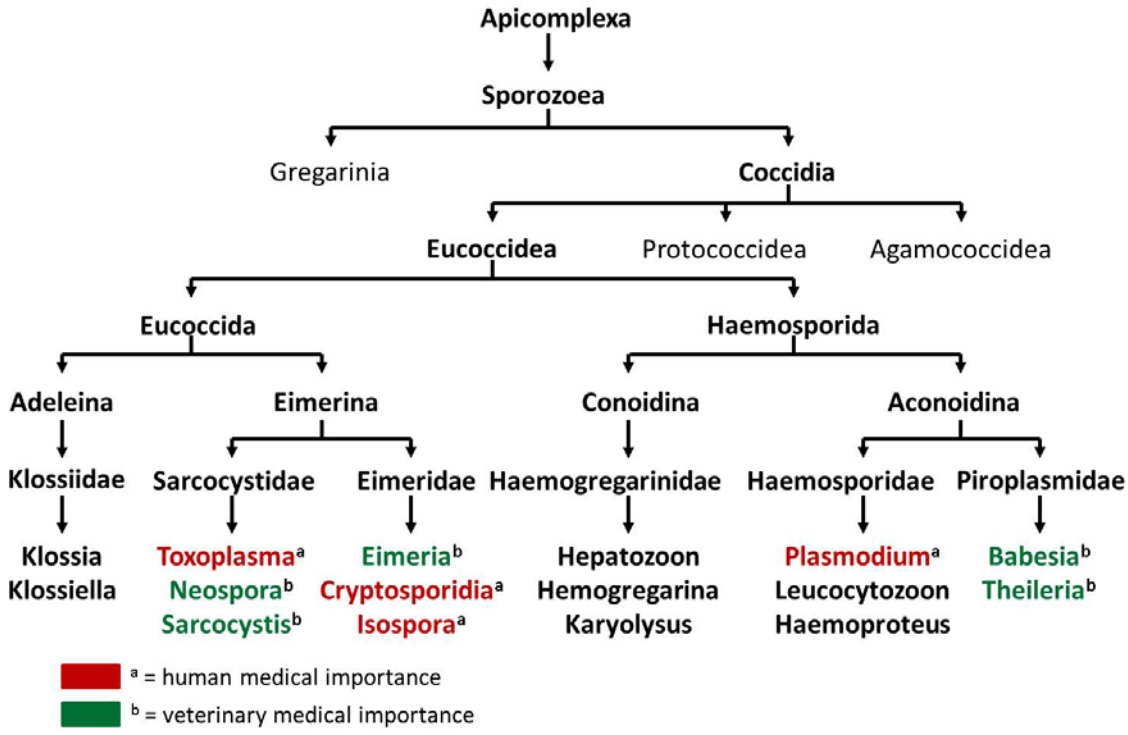

Figure 1.1. Phylogenic tree of Sporozoea (Apicomplexa/Alveolata). *Sarcocystis neurona* belongs to the family Sarcocystidae, along with *Toxoplasma*, and *Neospora*. Many apicomplexan parasites have human and veterinary medical importance. Modified from Beck et al., 2009.

Sarcocystis neurona has a two host predator-prey life cycle, consisting of a definitive and intermediate host (Dubey, 1976) (Figure 1.2). The definitive host is the North American opossum (*Didelphis virginiana*) (Fenger et al., 1995) and the South American opossum (*Didelphis albiventris*) (Dubey et al., 2001a). The *S. neurona* life cycle begins with the definitive host (opossum) ingesting sarcocysts from muscle tissue of an infected intermediate host. Within the gastrointestinal tract of the definitive host, the organism undergoes sexual reproduction to form oocysts, each containing two sporocysts. Sporocysts are excreted in the feces, being immediately infective (Dubey, 1976). Intermediate hosts ingest the sporocysts via contaminated food and/or water sources. Within the intestines of the intermediate host, the sporocysts excyst, releasing sporozoites that penetrate intestinal epithelial cells. After undergoing asexual reproduction (schizogony), merozoites are released into the bloodstream and mature into sarcocysts in skeletal muscle tissue (Dubey, 1976).

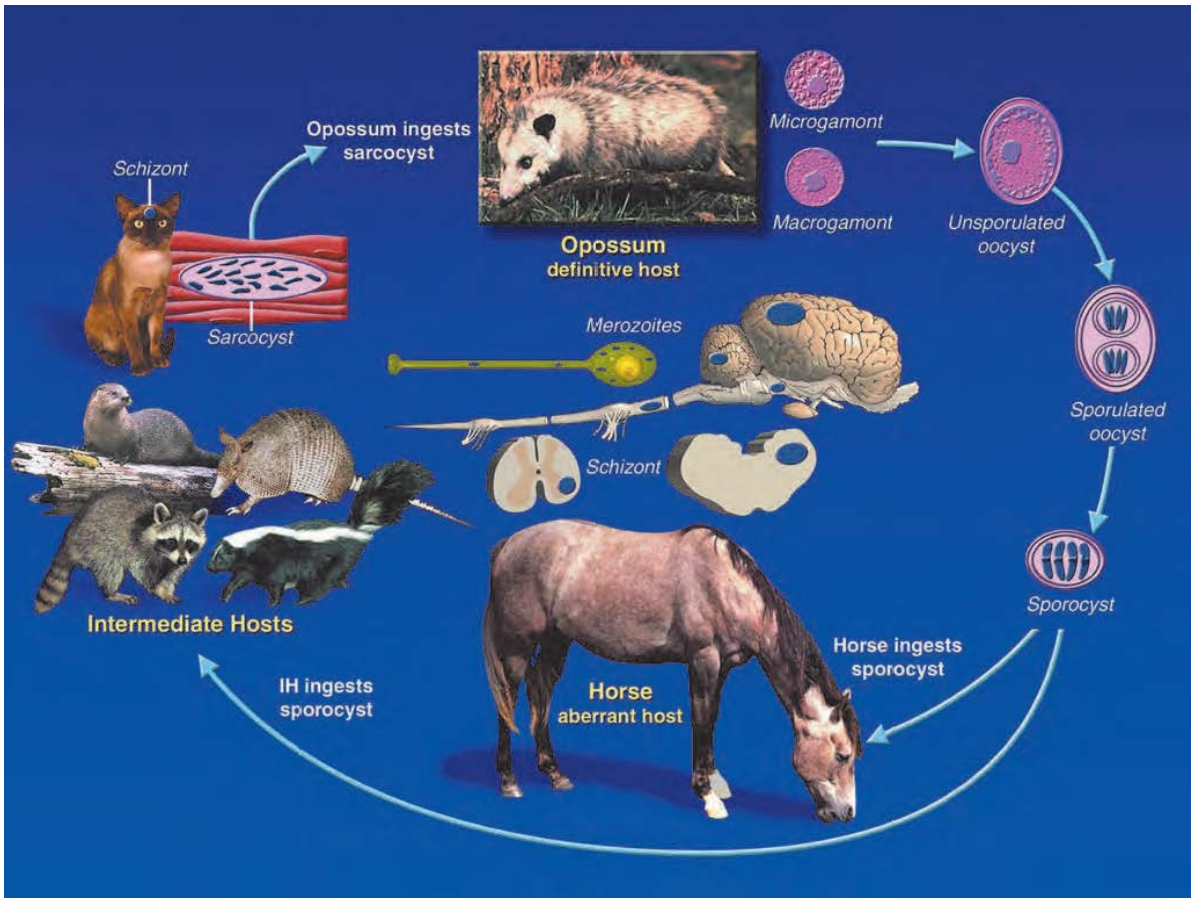

Figure 1.2. Life cycle of *Sarcocystis neurona*. The opossum, the definitive host, excrete sporocysts in feces. The intermediate host (IH) ingest sporocysts, which develop into sarcocysts within muscle tissue. The horse is an aberrant host ingesting sporocysts through contaminated feed. (Saville et al., 2002)

Completion of the *S. neurona* life cycle was accomplished using the domestic cat as an experimental intermediate host (Dubey et al., 2000). Several species have since been recognized as natural intermediate hosts, including the nine-banded armadillo (*Dasypus novemcinctus*) (Cheadle et al., 2001a), the striped skunk (*Mephitis mephitis*) (Cheadle et al., 2001b), the raccoon (*Procyon lotor*) (Stanek et al., 2002), the domestic cat (*Felis*

domesticus) (Stanek et al., 2003), and the sea otter (*Enhydra lutris*) (Dubey et al., 2001c). The horse is considered an aberrant or dead-end host, since *S. neurona* does not mature into sarcocysts in the muscles of infected horses (Dubey et al., 2001b). Therefore, horses are unable to infect a definitive host, resulting in an incomplete life cycle (MacKay, 1997a).

1.4. Pathogenesis

The pathogenesis of EPM in the horse is not thoroughly understood. Horses ingest food and/or water sources contaminated with *S. neurona* sporocysts, which travel to the intestine. The sporocysts excyst, releasing sporozoites, which invade the intestinal epithelium, progressively moving into the endothelium of blood vessels. The organisms asexually reproduce to form meronts, causing the endothelial cell to rupture, releasing merozoites into the bloodstream (Furr, 2006). The merozoites migrate to the CNS (MacKay et al., 2000), although the actual mechanism of transport into the CNS has yet to be determined. It is proposed that *S. neurona* passes through the blood-brain barrier via leukocytes or the cytoplasm of endothelial cells (Furr, 2006). By invading the leukocyte, *S. neurona* is not only provided access to the CNS, but protection from antibodies (Lindsay et al., 2006). Once inside the CNS, *S. neurona* invades neurons and microglial cells, and slowly undergoes additional asexual reproduction to form schizonts. Eventually, the structures rupture, releasing merozoites, each of which can repeat the reproductive process (Simpson and Mayhew, 1980).

Several factors likely affect the progression of the disease, including the number of organisms (Sofaly et al., 2002), length of time before treatment (Saville et al., 2000a), location of lesion (Dubey et al., 2001b), and stressful events during infection (Saville et al., 2001).

1.5. Clinical Signs

The clinical signs of EPM are extremely variable due to the possibility of *S. neurona* causing lesions anywhere in the CNS. Some horses present with clinical signs that appear gradually over time, while others display a much faster progression (Reed, 2008). Early signs of EPM are frequent stumbling and unexplainable lameness of the thoracic or pelvic limbs. The classic EPM clinical signs are asymmetrical ataxia (incoordination) with focal muscle atrophy (Dubey et al., 2001b).

Horses with an affected spinal cord exhibit gait abnormalities, manifested as ataxia of the limbs, generally affecting one side more than the other. This leads to asymmetrical muscle atrophy of the shoulder or rump (Reed, 2008). Damage to the grey matter results in focal muscle atrophy and severe muscle weakness; white matter damage manifests as ataxia and weakness in limbs caudal to the site of damage (MacKay et al., 2000). Additional signs may include dragging of the hoof especially while turning, hypometria of forelimbs, and reluctance to back up. Some clinical signs may only be witnessed while the horse is in training, such as head tossing, inability to maintain a lead, and a sore back (MacKay, 1997a). In rarer EPM cases involving an affected brain, clinical signs may include behavioral changes, blindness, seizures, drooping lip or ear, head tilt, and atrophy

of the tongue and the muscle of mastication (Furr et al., 2002). The disease can progress to the point of recumbency (MacKay, 1997a).

1.6. Pathology

Lesions associated with EPM are limited to CNS tissue (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974). The most commonly affected area of the brain is the brain stem, however, the majority of affected tissue is found in the spinal cord (Dubey et al., 2001b). Gross lesions are often visible in the grey matter of the spinal cord (MacKay et al., 2000). Histologically, characteristic lesions are easily recognizable (Beech and Dodd, 1974). Lesions are multifocal asymmetrical areas of hemorrhage, nonsuppurative inflammation, and necrosis (Dubey et al., 2001b). Inflammation of tissue is extremely variable with a mixture of cell types present, including lymphocytes, neutrophils, eosinophils, multinucleated giant cells, and gitter cells (Dubey et al., 2001b). Other common findings include perivascular cuffing of blood vessels with mononuclear cells, phagocytosis of axons with gitter cell formation, and astrocyte proliferation (MacKay et al., 2000). The white matter of diseased spinal cord tissue is often vacuolated due to the swelling and degeneration of axons (Beech and Dodd, 1974).

Histologically, *S. neurona* merozoites are difficult to distinguish from nuclear debris within areas of necrotic tissue with the use of hematoxylin and eosin (H&E) staining (Beech and Dodd, 1974). Immunohistochemical (IHC) staining, however, has been shown to aid in locating and positively identifying *S. neurona* (Hamir et al., 1993). Schizonts and merozoites can be located in neurons, giant cells, neutrophils, and

macrophages, with some merozoites found free in the tissue (Dubey et al., 2001b; MacKay et al., 2000).

1.7. Diagnosis

Diagnosis of EPM is often challenging. Not only do clinical signs mimic those of other diseases, but antemortem diagnostic tests are not always conclusive. Positive serum tests indicate that a horse has been exposed to *S. neurona*. A positive cerebrospinal fluid (CSF) test implies that the parasite has crossed the blood-brain barrier and is suggestive of an active infection (Furr, 2006).

1.7.1. Differential Diagnosis

Due to the wide range of potential clinical signs, EPM can present as other neurological diseases (MacKay et al., 2000). A thorough clinical and neurologic exam is necessary to rule out other neurological conditions (Furr et al., 2002). Cervical vertebral malformation (CVM) and Equine herpesvirus-1 myeloencephalopathy often result in symmetrical neurological signs (Mayhew, 1999). Ancillary testing can provide additional information to rule out other diseases. For example, if cervical spinal cord damage is suspected, cervical radiographs can aid in diagnosing other possible abnormalities, such as CVM, cervical fracture, or cervical osteoarthritis (Mayhew, 1999). If cervical spinal cord compression is suggested, a myelogram would definitively rule out CVM (Furr et al., 2002). Analysis of CSF is useful in distinguishing between viral and bacterial meningoencephalitis, as well as CNS trauma (Furr, 2006). The CSF can be tested for red

blood cell concentration, as well as for cytologic evaluation (Furr, 2006). An EPM horse has a normal CSF cytology, while West Nile Virus infected horses have abnormal CSF cytologies (Furr et al., 2002).

1.7.2. Antemortem Diagnosis

Several antemortem diagnostic tests recognize antibodies against *S. neurona*. The immunoblot (Western Blot) was the first test developed for detecting *S. neurona*-specific antibodies in the serum and CSF (Granstrom et al., 1993). In 2000, the Western blot was modified, increasing the sensitivity and specificity by using a bovine serum to block proteins nonspecific for *S. neurona* (Rossano et al., 2000). Another diagnostic test, the Indirect Fluorescent Antibody Test (IFAT) (Duarte et al., 2003; Duarte et al., 2004), is unable to differentiate between *S. neurona* and *Sarcocystis fayeri* infections. This is not problematic for diagnosis if clinical signs are taken into account, as *S. fayeri* is nonpathogenic in the horse (Saville et al., 2004a). Another diagnostic tool is the enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to the *S. neurona* surface antigen-1 protein (SnSAG1) (Ellison et al., 2003). Studies show that the SnSAG1 ELISA has low sensitivity and is not a useful diagnostic tool (Hoane et al., 2005; Johnson et al., 2010). This is understandable because not all strains of *S. neurona* express SnSAG1 (Howe et al., 2008). The development of an ELISA for SnSAG2, SnSAG3, and SnSAG4 are much more promising and have increased sensitivity and specificity (Yeargan and Howe, 2011). While the Western blot only detects the presence of antibodies, the IFAT and ELISA have increased diagnostic value since they are able to

quantify the amount of antibody present in samples with end-point titers (Duarte et al., 2003; Hoane et al., 2005).

Testing paired samples of serum and CSF is useful in the diagnosis of EPM (Furr, 2006). Passive transfer of antibodies from the blood through the blood-brain barrier results in the presence of antibodies in the CSF (Furr et al., 2011). Normally, the amount of antibody in the CSF is proportionate to the amount in the serum (Furr, 2002). However, during an active CNS infection, antibodies will be produced resulting in a higher amount of antibody in the CSF than would be proportionally expected (Furr et al., 2011). However, blood contamination of the CSF can produce a false-positive test result (Finno et al., 2007; Miller et al., 1999). Two CSF indices used to determine the CSF/serum proportions are the Goldman-Witmer coefficient (C-value) and the antigen-specific antibody index (AI) (Furr et al., 2011).

An additional tool for EPM diagnosis is the horse's response to anti-protozoal treatment. If the horse responds favorably to treatment, it potentially suffered from EPM. With this said, even in the event of diagnosis of EPM it is inconclusive as other diseases may respond to this type of treatment (Bentz et al., 1999).

1.7.3. Postmortem Diagnosis

The "gold standard" (Duarte et al., 2003) and only definitive diagnosis of EPM is a postmortem microscopic identification of *S. neurona* in the brain or spinal cord (Furr et al., 2002). Unfortunately, organisms can be easily overlooked, as usually only a small number of organisms are located in areas of inflammation and tissue necrosis (Dubey et

al., 2001b). The use of IHC staining with *S. neurona* antibody has been shown to aid in the detection of *S. neurona* by increasing the identification of organisms by 31% (20% identification with H&E staining; 51% with IHC staining) (Hamir et al., 1993). Prior treatment for EPM with anti-protozoal drugs decreases the likelihood of identifying *S. neurona* organisms in tissues (Boy et al., 1990). Due to these complicating issues, an EPM diagnosis is often based on the presence of characteristic lesions indicative of EPM, regardless of identification of *S. neurona* (MacKay et al., 2000).

1.8. Treatment

Time is the most crucial factor in the treatment of EPM. Earlier treatment will reduce the amount of CNS damage, which can be permanent (Dubey et al., 2001b). Medication will kill the protozoa, but will not repair the damage to the nervous tissue. If too severely damaged, the horse may need to be euthanized (Morley et al., 2001). Treatment is fairly expensive (Saville et al., 2000a), but usually leads to recovery in 70-75% of horses (Dubey et al., 2001b). There are several treatment options available for EPM.

1.8.1. Anti-protozoal Treatment

The Food and Drug Administration (FDA) has approved several treatments for EPM. Beginning in the 1970s, sulfadiazine/pyrimethamine was the traditional treatment (Mayhew et al., 1976). The drug, ReBalance, a combination of sulfadiazine and pyrimethamine (25%:1.25% suspension), has since been approved by the FDA (MacKay, 2006). ReBalance requires a prolonged administration of 90 to 270 days (Animal Health Pharmaceuticals, 2004). The drug limits the protozoan's ability to synthesize folic acid,

unfortunately, reducing the host animal to do so as well (MacKay et al., 2000). Long term treatment may cause anemia. Supplementation with folic acid is rarely necessary if the horse has access to high quality green forage (i.e., alfalfa hay) (MacKay, 1997a). The drug should not be administered to pregnant mares, as it has been known to cause abortions (MacKay, 2006).

The anticoccidial drug, Marquis, a 15% ponazuril paste, is another drug option. This drug targets *S. neurona*, while leaving the host tissue unaffected (Bayer Corporation, 2001). Marquis is administered orally, once daily, for 28 days and appears to be a safe course of treatment when used at the recommended dosage (Bayer Corporation, 2001). A third drug, Navigator, a 32% nitazoxanide paste, is administered orally for 28 days (IDEXX Pharmaceuticals, 2003). Navigator is FDA-approved, but the manufacturer, IDEXX Pharmaceuticals, stopped production of this drug in 2009. This drug has a broad spectrum of activity against parasites, bacteria and viruses, including the natural bacterial flora of the horse's gastrointestinal system (MacKay, 2006). Consequently, a horse on Navigator needs to be closely monitored for signs of toxicity, such as colic and diarrhea (MacKay et al., 2000). The newest drug option is Protazil, a 1.56% pellet of diclazuril (Schering-Plough Animal Health, 2007). This drug is an anticoccidial and considered relatively safe for the host tissue (MacKay, 2006). These top-dress pellets are administered for 28 days (Schering-Plough Animal Health, 2007).

1.8.2. Ancillary Treatment

Additional therapies are sometimes recommended to reduce inflammation and minimize further CNS damage (MacKay, 1997a). As *S. neurona* merozoites rapidly die in response

to anti-protozoal therapy, an inflammatory response can occur within the CNS. Nonsteroidal anti-inflammatory drugs (NSAIDs) (i.e., flunixin meglumate) and dimethyl sulfoxide (DMSO) are recommended during the first 1-2 weeks of treatment (MacKay, 2006). Corticosteroids are usually avoided, as they may increase neurologic symptoms (Cutler et al., 2001). However, some horses exhibit increasingly severe clinical signs at the onset of treatment, and the use of a corticosteroid may help control the inflammatory response initiated by the anti-protozoal drug (MacKay et al., 2000). Vitamin E can be given throughout the course of anti-protozoal therapy to prevent further oxidative damage of the CNS and to promote healing (MacKay, 1997a). Previous research suggests that EPM horses exhibit a decreased cell-mediated immunity, in particular a Th-1 response (Tornquist et al., 2001). Thus, non-specific immune stimulants, such as levamisole, killed *Propionibacterium acnes*, and Mycobacterium wall extract are sometimes used in addition to anti-protozoal therapy (MacKay, 2006).

1.9. Prognosis

The prognosis of a horse diagnosed with EPM is dependent upon the severity of clinical symptoms and the response to treatment (Saville et al., 2000a). Sixty percent of moderately to severely affected horses usually improve after treatment, with 10-20% recovering completely and are highly prone to relapse. Eighty percent of mildly affected horses will improve, with 50% recovering completely and being less prone to relapse (MacKay, 2006).

Horses treated earlier are more likely to recover completely (Saville et al., 2000a). Treated horses are 10 times more likely to improve than are horses left untreated (MacKay et al., 2000). A relapse, or the reoccurrence of clinical signs once treatment has ended, is estimated to occur in 10% of horses within 3 years of discontinuing treatment (MacKay, 2008).

1.10. Epidemiology

Prevalence of EPM is dependent upon the geographic distribution of the opossum (*D. virginiana* and *D. albiventris*), which is limited to North, Central, and South America (Dubey et al., 2001a; Fenger et al., 1995). Reports of EPM cases in the eastern hemisphere are occasionally identified in horses that originated from the Americas (Lam et al., 1999; Mayhew and Greiner, 1986; Ronen, 1992). The United States Department of Agriculture (USDA) reports that only 14 of 10,000 horses are actually diagnosed with EPM each year in the United States (US) (National Animal Health Monitoring System (NAHMS), 2001). This number is significantly smaller than the estimated 50% exposure rate of horses in the US (MacKay, 1997b). Seroprevalence of *S. neurona* varies throughout the country: 89.2% in Oklahoma (Bentz et al., 2003), 53.6% in Ohio (Saville et al., 1997), 45.3% in Chester County, Pennsylvania (Bentz et al., 1997), 45% in Oregon (Blythe et al., 1997), and 33.6% in Colorado (Tillotson et al., 1999). Variation of seroprevalence within each region is affected by different climatic factors. Oregon exhibited a higher seroprevalence in wetter coastal regions than in more arid regions

(Blythe et al., 1997). Ohio showed an association between a decrease in seroprevalence and the number of days the temperature was below freezing (Saville et al., 1997).

Several risk factors have been associated with the development of EPM. While seroprevalence increases with the age of the horse (Bentz et al., 2003; Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Tillotson et al., 1999), younger horses (1-5 years old) are at a higher risk for actually developing EPM (Boy et al., 1990; Cohen et al., 2007; Saville et al., 2000b). Certain breeds, such as Standardbred and Thoroughbred, appear to have a higher incidence of EPM (Boy et al., 1990; Rooney et al., 1970). These breeds are often associated with intense training at an early age, which could compromise their immune system. Horses involved in racing or competitions have an increased risk for EPM (Cohen et al., 2007; Saville et al., 2000b). Other stressful events that may be associated with EPM include injury/accident, surgery, and parturition (Saville et al., 2000b). The occurrence of EPM increases during the spring, summer and fall (Saville et al., 2000b), which may be due to a climatic effect (Saville et al., 1997) or an increased amount of travel and competitions during these months. The presence of the opossum directly correlates with EPM cases, as the incidence of EPM is greater in areas that contain higher populations of opossums (MacKay et al., 2000). The risk of EPM is greater if wooded areas surround the farm and lower if a creek or river is close to the property (Saville et al., 2000b).

1.11. Prevention

Certain management practices can be followed to decrease horse exposure to *S. neurona*. Trapping or keeping opossums away with specialized fencing (MacKay, 1997a) will prevent them from accessing the food and water sources of the horse (Saville et al., 2000b). Keeping grain in securely closed containers and cleaning up any spilled grain, fallen fruit, or bird seed will eliminate the opossums' food sources (MacKay, 2006), as well as storing forage (hay) in a facility that excludes wildlife (MacKay et al., 2000). Providing water sources for horses, such as water troughs, that are separate from ponds or creeks removes them from access to wildlife. Removing carcasses from the property and disposing of them properly prevents scavenging by opossums (Saville et al., 2002).

The use of an anti-protozoal drug as a daily preventative has been proposed (Saville et al., 2002). Protazil (diclazuril) appears promising, as it easily dispenses as a top-dress to horse feed (Schering-Plough Animal Health, 2007). However, it is unknown how a daily preventative affects the horse's natural immune response to *S. neurona*. Ultimately, this option is not cost-effective (MacKay, 2006). Another preventative option is the use of the drug, Marquis (ponazuril), intermittedly or during times of stress (Furr et al., 2006).

Fort Dodge Animal Health manufactured a vaccine from killed, cultured *S. neurona* merozoites in a MetaStim® adjuvant (MacKay, 2006). The USDA gave the vaccine a conditional license in 2000. The efficacy of the vaccine remained to be determined, as there was little data to support the effectiveness of the vaccine in preventing EPM (Marsh et al., 2004). As a result, the vaccine was removed from the market in 2009.

1.12. Cell-mediated Immunity

Cell-mediated immunity (CMI) is important for the elimination of intracellular parasites (Gazzinelli et al., 1994; Khan et al., 1997). To understand the role of the immune system in *S. neurona* infection, studies were initially conducted on mice. When injected subcutaneously with *S. neurona* merozoites, immunocompetent mice did not develop neurologic disease, nor were there any lesions in CNS tissue (Witonsky et al., 2003b). While there was an initial increase in the percentage of CD8+ peripheral blood lymphocytes and CD8+ splenocytes, levels returned to normal by the end of the 28 day study. This suggests that the immune response from a healthy immune system can prevent disease. CD8+ T cells seem to be involved in preventing disease in mice, as CD8 cell knockout (KO) mice inoculated with *S. neurona* merozoites developed meningo/encephalomyelitis (Witonsky et al., 2005).

Interferon-gamma (IFN-γ), a cytokine secreted by T cells (CD8+ and CD4+), natural killer (NK) cells, and IFN-γ producing non-T cells (Suzuki, 2002), has been shown to play a role in preventing neurologic infection. When infected with *S. neurona*, IFN-γ KO mice quickly developed meningo/encephalitis (Dubey and Lindsay, 1998; Witonsky et al., 2003a). Neurologic disease did not occur when severe combined immunodeficiency (SCID) mice were infected with *S. neurona* (Marsh et al., 1997). SCID mice lack adaptive immune responses from specific B and T cells, but have functioning NK cells. It is thought that the NK cells in SCID mice produce enough IFN-γ to protect them from neurologic disease (Sellon et al., 2004a). SCID mice treated with anti-IFN-γ antibody rapidly developed severe neurologic disease, highlighting the importance of this cytokine (Sellon et al., 2004a).

Limited research has been conducted on the CMI response in EPM horses. Studies used both naturally and experimentally infected horses. Initial research has shown that EPM horses have reduced cell-mediated responses to antigen-specific mitogens (Spencer et al., 2005; Spencer et al., 2004; Tornquist et al., 2001). It is difficult to determine if the immunosuppression is a result of the parasite suppressing the horse's immune response, or rather if the condition existed prior to the infection and is essential for the development of the disease (Tornquist et al., 2001). Both naturally and experimentally infected horses showed a suppressed response to the non-antigen specific mitogen, phorbol myristate acetate/ionomycin (PMA/I), in vitro (Witonsky et al., 2008; Yang et al., 2006). Compared to normal horses, EPM horses have increased interleukin (IL)-4 expression (Spencer et al., 2005) and suppressed IFN-γ mRNA expression in lymphocytes (Spencer et al., 2004). Studies show contrasting results regarding immune cell subsets. When compared to non-EPM horses, Tornquist et al. (2001) saw a decrease in CD4+ cells, while Yang et al. (2006) documented an increase in CD4+ cells. Witonsky et al. (2008) was the only study to note a decreased percentage of CD8+ cells. As these studies were completed *in vitro*, they likely do not represent the actual immune response within the horse.

1.13. Infectious Disease Susceptibility

The interaction between environmental and genetic factors is responsible for the development of infectious disease (Casanova and Abel, 2005). Exposure to infectious pathogens is critical for the progression of disease, while genetic factors affect an individual's susceptibility to disease (Kwiatkowski, 2000). Genetic predisposition to

infectious disease is considered to be either monogenic or polygenic (Alcais et al., 2009). Monogenic, or primary immunodeficiencies (PID), predisposition results from a single gene mutation causing susceptibility to a single disease (Casanova and Abel, 2007). Monogenic diseases have rare susceptibility alleles, display a high penetrance with a severe phenotype, and follow a Mendelian inheritance pattern (Casanova and Abel, 2005). Examples of PIDs include Mendelian susceptibility to Mycobacterial disease (MSMD) and pyogenic bacterial infections. Children suffering from MSMD are susceptible to weakly virulent Mycobacteria, but show resistance to most other infectious agents (Alcais et al., 2005). These children have a mutation in the IL-12-IFN-γ pathway (Dorman and Holland, 2000; Newport et al., 1996). Pyogenic bacterial infections result from a susceptibility to *Streptococcus pneumoniae* due to an interleukin-1 receptor-associated kinase (IRAK)-4 deficiency (Picard et al., 2003).

The majority of infectious diseases are complex. A combination of multiple genes are involved in polygenic predisposition with each gene contributing slightly to the overall susceptibility (Alcais et al., 2009). Complex diseases have common susceptibility alleles and do not follow the Mendelian pattern of inheritance (Casanova and Abel, 2005). Schistosomiasis was the first infectious disease to have mapped a susceptibility locus. This locus controls the level of infection with the parasite *Schistosoma mansoni* (Marquet et al., 1996). Diseases, such as leprosy (Abel and Demenais, 1988) and pulmonary tuberculosis (Alcais et al., 2005), display a polygenic predisposition. Variants in the regulatory region of PARK2 and PACRG have been associated with leprosy susceptibility (Mira et al., 2004). Pulmonary tuberculosis susceptibility has been linked to the chromosomal region 8q12-q13 (Baghdadi et al., 2006), as well as a mutation in the

promoter region of the monocyte chemoattractant protein-1 (MCP1) (Flores-Villanueva et al., 2005).

The genetic background of mouse strains affects their susceptibility to disease. Thus, Balb/c mice are resistant to the coccidium *Eimeria ferrisi*, while C57BL/6 mice are susceptible to this infection (Klesius and Hinds, 1979). Different susceptibility of mice strains was also evidenced with *T. gondii* infection. At a high dose (1×10^5 *T. gondii* trophozoites), Balb/c and DBA/2 mice were most susceptible to infection, while DBA/1 and white SW/SIM strains were the most resistant. However, a lower dose (1×10^3 *T. gondii* trophozoites), Balb/c mice became one of the more resistant strains (Araujo et al., 1976). The variation of survival between the mice strains seems to be dependent upon genes of the major histocompatibility complex (MHC), as well as non-MHC genes (Deckert-Schluter et al., 1994; Williams et al., 1978).

1.14. Genome-wide Association Study

Genome-wide association studies (GWAS) have become a powerful approach in identifying genes involved with monogenic and complex traits (McCarthy et al., 2008). Genome-wide association studies scan the entire genome for common variants, and usually compare a group that has a certain trait (i.e., disease) with a control group that does not exhibit the trait (Pearson and Manolio, 2008).

Single nucleotide polymorphisms (SNP) are the most common sequence variant (Collins et al., 1998), and are used as markers throughout the genome (Pearson and Manolio, 2008). These variants create diversity in populations, as well as being responsible for an

individual's disease susceptibility. Most SNPs do not affect the function of the gene, instead they aid in locating other mutation(s) that are impacting gene function. SNPs that do modify functionality of the gene are usually located in the regulatory or coding regions of the genome (Collins et al., 1998). SNP chips are tools that can rapidly analyze the genome for SNPs and map them to specific regions on chromosomes (Chowdhary et al., 2008). These arrays have probes that target thousands of SNPs, which uniformly span the entire genome. This high-throughput genotyping technology is now commercially available (Chowdhary and Raudsepp, 2008).

A GWAS typically employs four steps. First, a sample set is chosen, consisting of individuals displaying a specific trait or disease. A comparison group is necessary that does not exhibit the trait or disease of interest (Wellcome Trust Case Control Consortium, 2007). For all individuals, DNA is extracted and genotyped. Statistical tests are used to assess for any association between SNPs and the trait or disease of interest (Pearson and Manolio, 2008). Finally, a technical validation is necessary, as well as a replication of the study in an independent population, to ensure that the results represent valid associations (McCarthy et al., 2008).

Through the use of GWAS, loci have been identified that implicate predisposition to disease (McCarthy et al., 2008). Such diseases include type 1 (Hakonarson et al., 2007) and type 2 diabetes (Steinthorsdottir et al., 2007), inflammatory bowel disease or Crohn's disease (Hampe et al., 2007), rheumatoid arthritis (Begovich et al., 2004), and behavioral disorders, such as schizophrenia and bipolar disorder (Craddock et al., 2005). Susceptibility to infectious diseases is usually polygenic and considered a complex trait, as several genes are generally involved (Bellamy, 2006). One of the first infectious

disease GWAS was performed in 2007, discovering genetic determinants that affect the viral load during asymptomatic stages of HIV (Fellay et al., 2007). Another early infectious disease GWAS was performed on Kawasaki disease, finding several variants in genes that are involved with the pathogenesis (Burgner et al., 2009). Knowledge of affected genes can help identify pathways involved with disease susceptibility, increase understanding of the pathogenesis, and aid in the development of potential therapeutics (Davila and Hibberd, 2009).

The technology used in conducting GWAS in humans is now being applied in other species, such as the cow and horse. Sequencing the genome of these animals was instrumental in the development of tools specific for analyzing their genomes, making the study of complex traits and diseases possible (Chowdhary and Raudsepp, 2008). In cattle, GWAS have been used heavily for improvement of production, conformation, and fitness. In the dairy industry, GWAS has been instrumental in breeding animals that have increased milk production (Wiggans et al., 2011). In the past, equine researchers have used gene maps and comparative genomics to identify monogenic traits, such as metabolic disorders (Dranchak et al., 2007), hereditary skin abnormalities (Tryon et al., 2007), and immune system disorders (Shin et al., 1997). The equine SNP chip has been implemented in numerous studies of more complex traits and diseases, including recurrent laryngeal neuropathy (Dupuis et al., 2011), osteochondrosis (Lykkjen et al., 2010), dwarfism (Orr et al., 2010), and equine viral arteritis (Go et al., 2011). This technology has also been used to develop optimum racing distance for Thoroughbreds (Hill et al., 2010) and desired coat colors (Reissmann et al., 2007).

1.15. Equine EPM Model

The development of an equine model of EPM would significantly enhance knowledge of the disease. An equine model would aid in understanding the pathogenesis of EPM and lead to potential improvements in the diagnosis, treatment, and prevention of the disease. Several studies have attempted to induce EPM with varying levels of success, utilizing three different methods to introduce *S. neurona* into the horse.

1.15.1. Intragastric Introduction

Intragastric introduction was the first method used to create an EPM model. Two approaches were used in the preparation of *S. neurona* sporocysts inoculums. In one approach, the sporocysts were collected from intestinal scrapings of naturally infected opossums (Fenger et al., 1997). Since the opossum is the definitive host for three *Sarcocystis* spp (*S. neurona, Sarcocystis falcatula, Sarcocystis speeri*) (Rosenthal et al., 2001), the inoculums were not pure *S. neurona*, but a mixture of *Sarcocystis* spp (Fenger et al., 1997). Bioassay or polymerase chain reaction (PCR) characterized the *S. neurona* inoculums from opossum's intestinal scrapings (Cutler et al., 2001; Saville et al., 2001). In a second approach, sporocysts were collected from laboratory-raised opossums. In this approach, tongues of naturally infected raccoons were fed to laboratory-raised opossums whose intestines were scraped for sporocysts, which were then fed to laboratory-raised raccoons. The raccoon muscle was subsequently fed to laboratory-raised opossums, from which the sporocysts were finally collected (Sofaly et al., 2002).

The initial study by Fenger et al. (1997) introduced an inoculum of mixed *Sarcocystis* spp sporocysts from the intestines of naturally infected feral opossums directly into the foals'

stomachs using a nasogastric tube. One to three doses of at least 2 x 10^6 sporocysts were administered to the foals. All foals seroconverted between 24 and 42 days post inoculation (dpi) and had CSF antibodies present between 24 and 42 dpi. Foals displayed mild neurologic symptoms, with 3 of the 5 foals exhibiting histopathologic lesions in the brainstem and/or spinal cord. However, no organisms were observed in the tissues (Fenger et al., 1997).

In a second study, Cutler et al. (2001) corticosteroids were used as an immunomodulator to induce EPM. An inoculum of PCR-characterized *S. neurona* sporocysts, collected from the intestines of naturally infected feral opossums, was administered via a nasogastric tube to horses for 7 consecutive days at a dose of 5 x 10^5 *S. neurona* sporocysts. A treatment group was additionally administered 0.1 mg/kg of the corticosteroid, dexamethasone, daily beginning 7 days prior to the first inoculation through the termination of the study. All horses seroconverted in the blood and CSF, but the dexamethasone-treated horses immunoconverted in less time and showed increased clinical signs. Unfortunately, it was difficult to determine if the horses displayed neurologic signs from the *S. neurona* infection or signs of weakness from systemic disease caused by the immunosuppression (Cutler et al., 2001).

Saville et al. (2001) examined the effect of stress, specifically corticosteroids and transportation, on generating EPM in the horse. Bioassay characterized *S. neurona* sporocysts from the intestines of naturally infected feral opossums were administered in a single dose of 8 x 10^4 sporocysts via nasogastric tube to horses in 2 treatment groups. Horses were then transported for 55 hours to create stress. Upon arrival at the facility, one treatment group was immediately administered *S. neurona* sporocysts. A second

treatment group was allowed 14 days to acclimate, and then given 0.5 mg/kg dose of dexamethasone prior to being inoculated with 8 x 10^4 *S. neurona* sporocysts. This group continued to receive 0.2 mg/kg of dexamethasone twice a week for the remainder of the study. While all horses immunoconverted in both serum and CSF, transportation resulted in a quicker seroconversion time (Saville et al., 2001). Additionally, dexamethasone-treated horses presented milder clinical signs, which contradicts previous study results from Cutler et al. (2001). Since transportation stress seemed to be effective in inducing EPM, Saville et al. (2004b) conducted another study to test the effect of a second transportation. The inoculums of *S. neurona* sporocysts were taken from laboratory-raised opossums. Horses underwent prolonged transportation and then were inoculated with a single dose of 1.5 x 10^6 *S. neurona* sporocysts. One treatment group was not transported again, while a second treatment group was transported again at either 4, 11, or 18 dpi. All horses seroconverted between 12 and 21 dpi. The treatment group with the single transportation exhibited more severe clinical signs than the horses with the second transport (Saville et al., 2004b).

Sofaly et al. (2002) studied the effect of inoculation doses on the induction of EPM. Inoculua of *S. neurona* sporocysts, derived from laboratory-raised opossums, were administered to horses in doses varying from 10^2 - 10^6. There was a dose-dependent relationship, as increased doses resulted in earlier seroconversion time. Results showed that a dose of at least 10^6 was necessary to consistently induce *S. neurona* infection (Sofaly et al., 2002).

The method of inducing EPM via *S. neurona* sporocysts to the stomach of the horse resulted in fairly consistent findings between studies. Horses had both serum and CSF

immunoconversion, mild to moderate clinical signs, and occasional lesions present in the brain/spinal cord tissue. However, this method was unable to reproduce severe clinical signs (i.e., asymmetrical signs, inability to rise) and *S. neurona* organisms were never recovered nor seen histopathologically.

1.15.2. Intrathecal Introduction

Intrathecal introduction was the second method used to create an EPM model. In a study by Lindsay et al. (2000), *S. neurona* merozoites were directly introduced into the CNS of the horse. In an effort to maintain a consistent CSF volume, 10mL of CSF were removed from the horses prior to injecting 5×10^6 culture derived *S. neurona* merozoites suspended in 10mL plasmalyte into the subarachnoid space. Horses did seroconvert and antibodies were present in the CSF, but no clinical signs of EPM were evident (Lindsay et al., 2000). Intrathecal introduction of *S. neurona* merozoites, therefore, did not prove to be a successful method to establish EPM.

1.15.3. Intravenous Introduction

The final method used to create an EPM model was through an artificial parenteral introduction of *S. neurona*. While investigating *S. neurona* parasitemia in SCID horses, Sellon et al. (2004b) injected intravenously 5×10^8 *S. neurona* culture derived merozoites into both SCID and immunocompetent horses. Neurological symptoms were present in 2 of the 3 immunocompetent horses. Immunocompetent horses were able to control parasitemia. Using PCR, *S. neurona* was not detected in visceral tissues, but was present in neural tissue. Conversely, none of the SCID horses developed any neurologic signs.

However, SCID horses were unable to control parasitemia, and *S. neurona* was detected in visceral tissues (Sellon et al., 2004b).

Ellison et al. (2004) developed a procedure to infect horses with host lymphocytes containing intracellular *S. neurona*. Blood was collected from the horse, and the buffy coat was isolated and incubated with *S. neurona* merozoites for 5 hours. The infected cells were added to 6mL of collected blood and injected intravenously back into the horse. A single horse was given 100,000 merozoites at four different times, each a week apart. Three other horses were dosed with varying amounts of merozoites (100, 1,000, 10,000) daily for 15 consecutive days. All horses developed moderate clinical signs, with blood and CSF immunoconversion by day 7. One of the challenged horses was a pregnant mare. Within the lung tissue of the fetus, the author states that an organism or artifact was identified with H&E staining and there was antibody binding using IHC staining (Ellison et al., 2004). It seems more likely that what the author viewed was actually an artifact, as *S. neurona* is not known to be present in the lung tissue.

A second study by Ellison and Witonsky (2009) modified this procedure. Blood from each horse was collected in an ethylenediaminetetraacetic acid (EDTA) tube, and 6,000 *S. neurona* merozoites were directly inoculated into each of the blood tubes. Blood tubes were incubated at 37°C overnight. The blood was then injected intravenously back into the horse, and the process was repeated for 14 consecutive days. This resulted in horses developing clinical signs with serum and CSF immunoconversion (Ellison and Witonsky, 2009).

The intravenous method of induction of EPM resulted in moderate clinical signs and earlier seroconversion times than the intragastric or intrathecal methods. Unfortunately, *S. neurona* has yet to be identified histologically within lesions of the CNS tissue.

1.16. Research Objectives

Although extensive EPM research has been conducted over the past two decades, there is still much to learn about this disease. The objective of the two studies in this thesis was to further understand factors involved in the development of EPM within the horse. The first study explored a possible genetic susceptibility to EPM, as only a small number of horses exposed to *S. neurona* actually develop the disease. The susceptibility was investigated by performing a genome-wide association study (GWAS) on formalin-fixed, paraffin-embedded (FFPE) tissues from definitively positive EPM horses. The second study tested the viability of a previously described artificial infection method to create a reliable equine EPM model. The development of a working EPM model would be useful in understanding the pathogenesis of the disease, and aid in the diagnosis, treatment, and prevention of EPM. Additionally, the immune response to the challenge infection was examined in the second study.

CHAPTER TWO

Genome-wide association study to investigate genetic susceptibility of equine protozoal myeloencephalitis

2.1. Introduction

Equine protozoal myeloencephalitis (EPM) is the most commonly diagnosed neurological disease of horses in the United States (Dubey et al., 2001b). The disease is caused by the protozoan parasite, *Sarcocystis neurona* (Dubey et al., 1991). Horses are an aberrant host of *S. neurona*, and are infected by ingesting feed or water contaminated with sporocysts. Within the horse, the organism infiltrates the central nervous system and initiates an immune response, which leads to damaged tissue and neurological disease (MacKay et al., 2000). Diagnosis of EPM is challenging, with microscopic identification of *S. neurona* in the brain or spinal cord during postmortem examination the only definitive diagnosis (Furr et al., 2002).

Seroprevalence studies show an exposure rate to *S. neurona* of approximately 50% in horses of the United States (MacKay, 1997b). However, only 0.5-1% of all horses are actually diagnosed with EPM (Dubey et al., 2001b). The factors involved in facilitating disease development are still unknown. Some breeds, such as the Thoroughbred and Standardbred, seem to have higher incidences of EPM (Boy et al., 1990; Rooney et al., 1970). Researchers have proposed a genetic basis for the susceptibility to EPM, but no studies have been performed to date. Investigating a potential genetic predisposition is now possible due to the availability of the equine genome sequence and technologies that

scan the genome to identify variants associated with susceptibility to disease (Wade et al., 2009).

The goal of this study was to perform a genome-wide association study (GWAS) of definitively positive EPM horses. This GWAS used archived formalin-fixed, paraffin-embedded (FFPE) tissues to identify potential genetic variants associated with disease susceptibility.

2.2. Materials and Methods

2.2.1. Case Selection

The case report database of the University of Kentucky Veterinary Diagnostic Laboratory (UK VDL) was searched to identify cases from 1993-2011 with a diagnosis of EPM. Each case report was reviewed for a statement of identification of protozoal organisms in CNS tissue. Archived hematoxylin and eosin (H&E) stained slides of the brain and spinal cord tissues from these cases were examined by light microscopy to confirm the presence of parasites.

2.2.2. Immunohistochemistry

Immunohistochemical (IHC) staining was completed using an automated staining system (Bond-maX, Leica Biosystems, Newcastle Upon Tyne, UK) following the manufacturer's IHC Protocol F and the Bond Polymer Refine detection system (Leica Biosystems). This procedure involved an automated dewaxing and rehydration of the tissue, continuing with

a heat-induced antigen retrieval using a ready-to-use citrate based buffer (pH 6.0) and surfactant (Leica Biosystems) at 100°C for 20 minutes. Slides were then incubated with 3% hydrogen peroxide (H_2O_2) for 5 minutes, followed by application of anti-*S. neurona* rabbit serum diluted 1:2500 with Bond Primary Antibody Diluent (Leica Biosystems), and incubated for 15 minutes. Slides were treated with postprimary blocking reagent for 8 minutes and then horseradish peroxidase-labeled IgG polymer for 8 minutes. Diaminobenzidine tetrahydrochloride (DAB) substrate was added, and the slides were incubated for 10 minutes. Finally, the slides were counterstained with hematoxylin for 5 minutes. Between each incubation step, slides were washed using Bond Wash Solution 10x Concentrate (Leica Biosystems), diluted with distilled water to a 1x working concentration, to remove any unbound material. Brain tissue from a clinical EPM horse with a large number of *S. neurona* organisms was stained as a control. A negative control consisted of only Bond Primary Antibody Diluent (Leica Biosystems).

Slides were removed from the instrument and washed under distilled water. Tissue was dehydrated using a graded series of alcohol washes (2-85% ethanol, 2-90% ethanol, 2-95% ethanol, 2-100% ethanol) and cleared with 4 xylene washes. Slides were covered using mounting medium and glass coverslips, and viewed with light microscopy to identify *S. neurona* organisms.

2.2.3. Genotyping

For each confirmed EPM case, 5 µm serial sections were cut from FFPE spleen/liver or cerebellum tissue. Five tissue scrolls from serial sections of each horse were placed in labeled Eppendorf tubes. Samples were sent to GeneSeek, Inc. (Lincoln, Nebraska) for

genotyping. DNA was isolated from tissues using a phenol/chloroform extraction method. Samples were run on a single Equine SNP50 BeadChip (Illumina, San Diego, California) using the manufacturer's protocols. This array contains 54,602 single nucleotide polymorphisms (SNPs) evenly distributed across all 31 autosomes with an average probe spacing of 43.2kb, derived from the EquCab2.0 SNP assembly of the horse genome (http://www.broadinstitute.org/mammals/horse). Average call rate was used for quality control.

2.3. Results

2.3.1. Case Selection

The UK VDL database contained 194 cases with a diagnosis of EPM, or suspected EPM, between 1993 and 2011. In 36 case reports, pathologists stated that organisms were observed in the CNS by histopathologic examination.

Signalment was obtained from each of the 36 case reports (Table 2.1). A majority of the horses were Thoroughbreds (n=29), other breeds included Quarter Horses (n=3), Tennessee Walking Horse (n=1), Percheron (n=1), and mixed breeds (n=1). There was minimal difference between the number of males (n=15) and females (n=19). Of the horses with a known age, about half (n=16) were 1-5 years old, while the remainder (n=15) were 6-30 years of age.

Table 2.1. Horses used in the study.

ID #	Year	Breed	Sex	Age	Parasites Identified – H&E	Parasites Identified - IHC
1	1993	Thoroughbred	Female	5	Yes	Yes
2	1993	Thoroughbred	Female	4	Yes	Yes
3	1994	Quarter Horse	Male	9	Yes	Yes
4	1994	Tennessee Walking Horse	Female	9	Yes	Yes
5	1994	Thoroughbred	Female	16	Yes	N/A[B]
6	1995	Thoroughbred	Male	2	Yes	Yes
7	1995	Thoroughbred	Female	3	N/A[A]	N/A[B]
8	1996	Thoroughbred	Unknown	Unknown	Yes	Yes
9	1997	Thoroughbred	Female	19	Yes	Yes
10	1998	Mixed Breed	Male	Adult	Yes	Yes
11	1998	Thoroughbred	Male	Unknown	Yes	Yes
12	1999	Thoroughbred	Female	Adult	Yes	No
13	2000	Thoroughbred	Female	6	Yes	Yes
14	2001	Thoroughbred	Male	15	Yes	Yes
15	2001	Quarter Horse	Female	3	Yes	Yes
16	2002	Thoroughbred	Male	2	Yes	Yes
17	2003	Thoroughbred	Female	3	Yes	Yes
18	2003	Thoroughbred	Female	5	Yes	Yes
19	2004	Thoroughbred	Female	3	Yes	Yes
20	2006	Thoroughbred	Male	1	Yes	Yes
21	2006	Thoroughbred	Female	13	Yes	Yes
22	2006	Thoroughbred	Male	3	Yes	Yes
23	2006	Thoroughbred	Male	12	Yes	No
24	2007	Quarter Horse	Male	8	Yes	Yes
25	2007	Thoroughbred	Male	3	Yes	Yes
26	2008	Thoroughbred	Male	18	Yes	Yes
27	2010	Thoroughbred	Unknown	1	Yes	Yes
28	2010	Thoroughbred	Male	1	Yes	Yes
29	2010	Thoroughbred	Female	30	Yes	Yes
30	2011	Thoroughbred	Female	7	Yes	Yes
31	2011	Thoroughbred	Female	Adult	Yes	Yes
32	2011	Percheron	Male	17	Yes	Yes
33	2011	Thoroughbred	Female	2	Yes	Yes
34	2011	Unknown	Female	14	Yes	Yes
35	2011	Thoroughbred	Female	11	Yes	Yes
36	2011	Thoroughbred	Male	2	Yes	No

[A] = No slides available at UK VDL
[B] = No tissue blocks available at UK VDL

2.3.2. Confirmation of Sarcocystis neurona

For all cases in which archived slides were available (n=35), parasites were visualized on H&E stained slides (Table 2.1). The infection presented differently across the cases. Lesions varied from focal areas of inflammation to severe inflammation with widespread necrosis (Figures 2.1A & 2.1B). Parasites were generally located within areas of inflammation (Figure 2.2A). The number of parasites present varied, but was usually associated with the degree of inflammation/necrosis. Thus, the more severe the inflammation, typically the higher number of parasites observed.

Immunohistochemical staining was used for further confirmation that the parasites present in the tissues were *S. neurona*, as IHC staining allows for identification of parasites, especially merozoites. The number of parasites located with IHC staining varied across cases. Within lesions, only a single *S. neurona* parasite was observed in some horses, while multiple *S. neurona* parasites in various stages of growth were seen in other horses (Figures 2.2B & 2.2C). Of the cases where tissue blocks were available (n=34), *S. neurona* parasites were identified in 31 cases (Table 2.1). Only a single parasite was observed in the corresponding H&E slide of the 3 cases that were not positive by IHC staining.

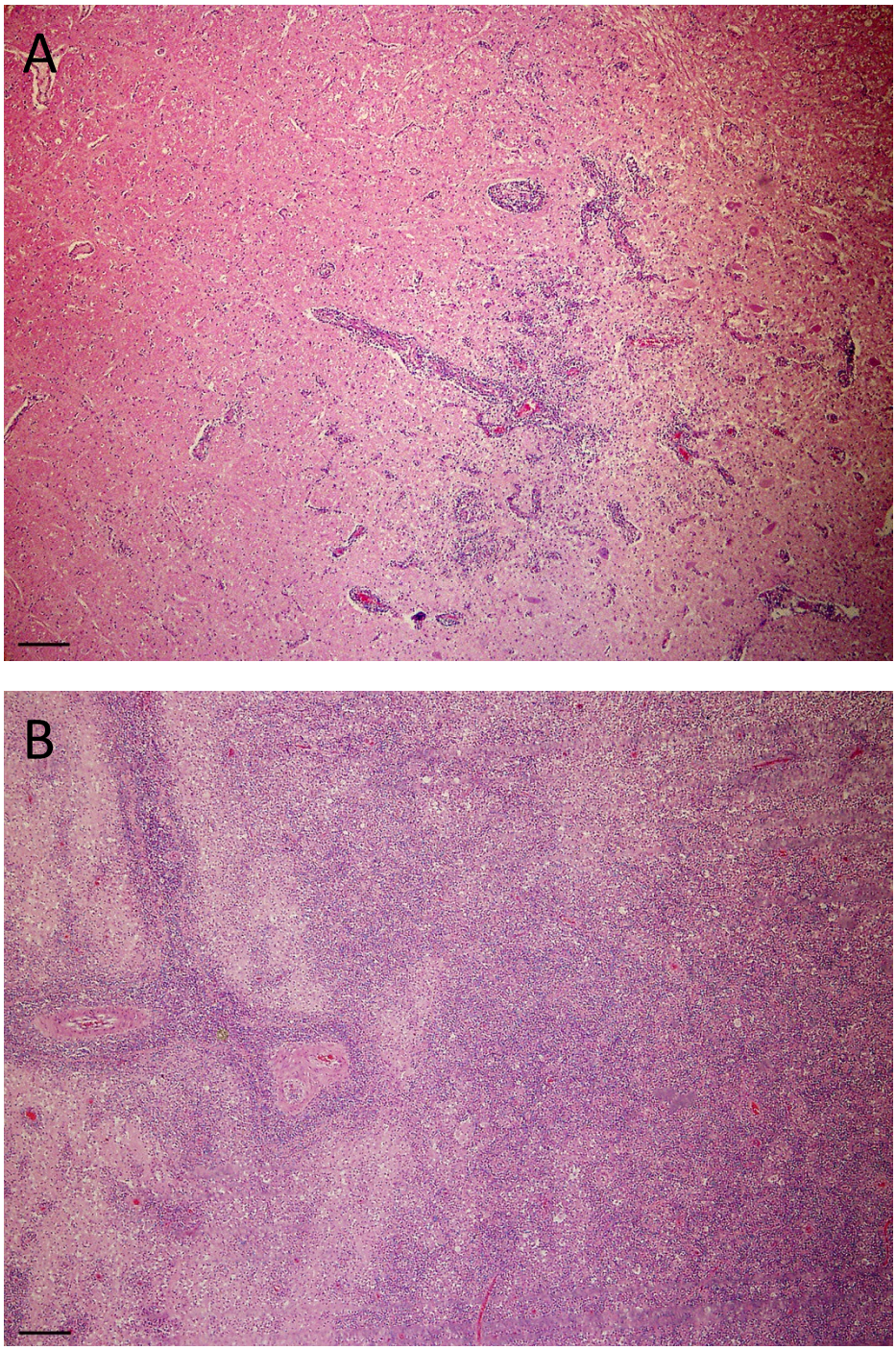

Figure 2.1. CNS lesions of EPM horses. Hematoxylin and eosin stain. Bar = 200µm. (A) Inflammatory foci within a spinal cord section of Horse 26, with a few perivascular cuffs containing inflammatory cells. (B) Severe inflammation present in brain tissue of Horse 24, with a heavy infiltration of monocytic cells and widespread tissue necrosis.

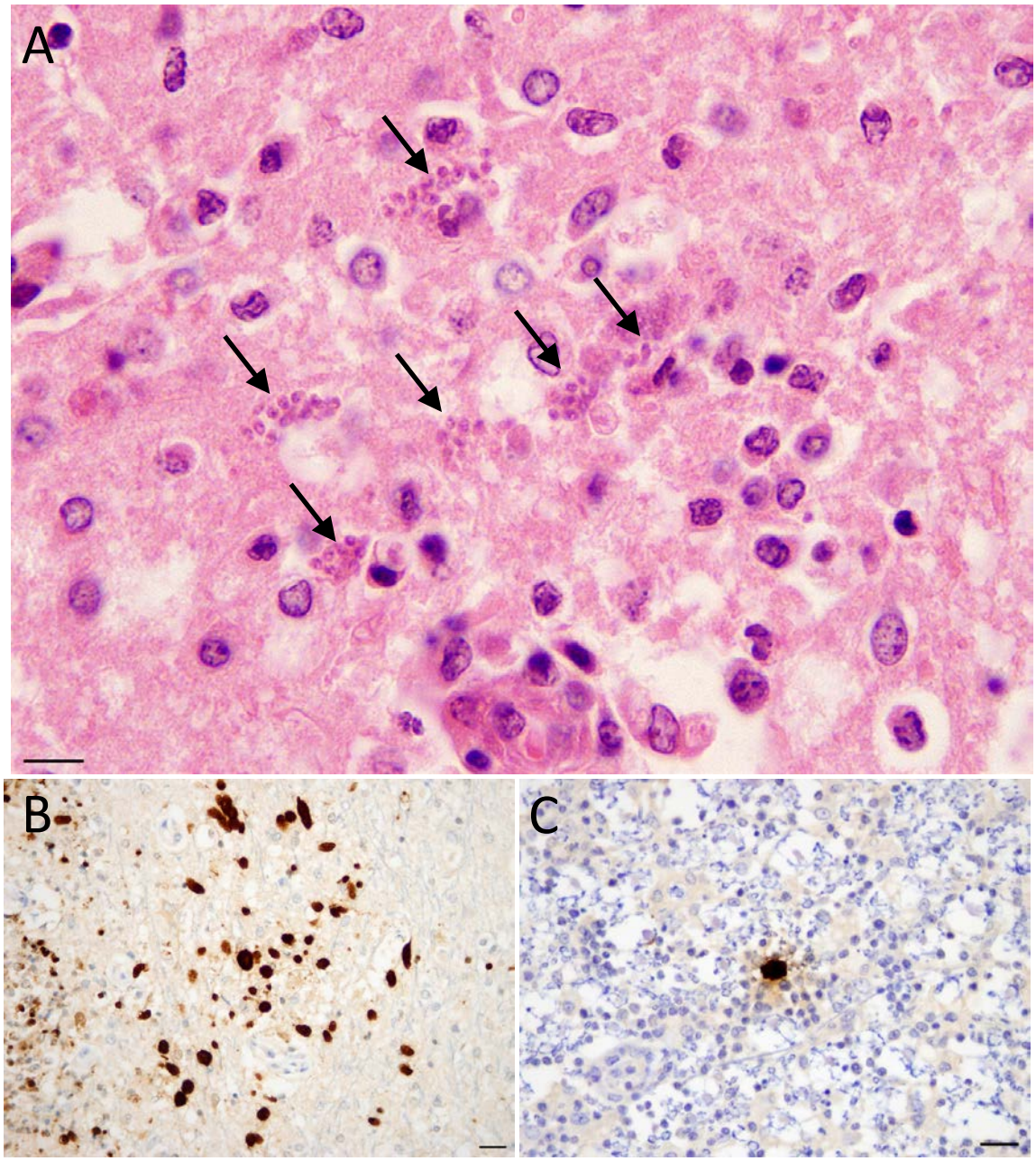

Figure 2.2. *Sarcocystis neurona* within CNS lesions of EPM horses. (A) Multiple *S. neurona* schizonts (arrows) within brain tissue of Horse 29. Hematoxylin & eosin stain. Bar = 10µm (B) - (C) Immunohistochemical staining with anti-*S. neurona* rabbit serum. Bar = 20µm. (B) Brain tissue with numerous *S. neurona* schizonts and merozoites of Horse 29. All brown staining indicates organisms. (C) A single *S. neurona* schizont located in the spinal cord of Horse 28.

2.3.3. Genotyping

Twenty-four definitive EPM cases of Thoroughbreds were selected to be genotyped and sent to GeneSeek, Inc. After purification from FFPE tissues, the majority of DNA samples were within optimal range (1.8-2.1) for purity (A260/280) (Table 2.2). The quantity of total DNA extracted from all 24 FFPE tissues was sufficient to run the SNP chip assay (>200ng) (Table 2.2). The range in the amount of DNA extracted was dependent on the size of the tissue present in the tissue block.

Only 5 of the samples genotyped had a call rate above 88% (Table 2.2) signifying that over 88% of the possible 54,602 SNPs were read (i.e. successful genotype identification). The tissues of these 5 cases were fixed in formalin for the shortest time period of all 24 cases analyzed. Call rates of the remaining 19 samples were only 43-68%. Any tissue fixed in formalin for more than 14 months resulted in a drop in call rate. The various tissue types from which DNA was extracted (i.e., adrenal gland, brain, heart, intestine, kidney, liver, lung, spleen, stomach, tongue) did not appear to effect the call rate.

Table 2.2. Call rate, DNA quantity and quality from FFPE tissue of EPM horses.

ID #	Month-Year	Call Rate	A260/280	Total DNA (ng)	FFPE Tissue[A]
35	Jun-11	0.9648756	2.00	1705.0	Adrenal gland, liver, spleen
31	Unknown-11	0.9298905	2.01	1878.0	Kidney, spleen
29	Nov-10	0.8959416	2.05	1744.5	Liver, spleen
33	Apr-11	0.8882084	2.04	3140.0	Kidney, spleen
30	Apr-11	0.8880847	1.73	2100.0	Spleen
6	Feb-95	0.6818547	2.03	1280.0	Liver, lung
9	Dec-97	0.6553452	1.94	3025.0	Liver, lung, spleen
19	Jan-04	0.6376516	2.05	1435.0	Kidney, liver, lung
1	Jun-93	0.6186742	2.54	948.5	Lung, spleen
16	Feb-02	0.6164161	2.59	1125.0	Liver, lung, spleen
28	Aug-10	0.5944538	2.02	620.5	Intestine, liver, spleen
21	Jul-06	0.5776262	1.75	2500.0	Liver, lung, spleen
11	Mar-98	0.5719500	1.88	1430.0	Liver, lung, spleen
2	Jul-93	0.5655778	1.96	1920.0	Liver, spleen
8	Oct-96	0.5600563	2.02	1227.0	Brain (Cerebellum)
17	Jan-03	0.5444042	2.05	3055.0	Heart, liver
20	Jun-06	0.5389445	1.84	747.5	Spleen, stomach
27	Apr-10	0.5227512	2.04	1117.0	Liver, spleen
18	Apr-03	0.5143684	2.08	1370.0	Brain (Cerebellum)
13	Jun-00	0.5122030	1.99	1941.5	Liver, lung, spleen
14	Feb-01	0.5084602	2.02	2980.0	Liver, lung, spleen
26	Jan-08	0.4909830	1.47	2930.0	Liver, lung, tongue
22	Sep-06	0.4555184	1.74	615.5	Lung, spleen
25	Oct-07	0.4347315	1.92	646.0	Spleen

[A] = Type of FFPE Tissue sent to GeneSeek

2.4. Discussion

A sample set of FFPE tissue was used for a GWAS of definitively positive EPM horses. The study was unsuccessful as the DNA was degraded from fixation. The samples with low call rates did not generate enough data for analysis as approximately half of their genomes were searched for sequence variants. With such limited data and no SNP chip

analysis possible, results were inconclusive if horses have a genetic susceptibility to EPM.

Archived FFPE tissues are a large resource for disease studies. Unfortunately, the formalin fixation used to preserve tissue morphology degrades DNA, producing DNA-interstrand and DNA-protein cross-links (Crisan and Mattson, 1993). Formalin can also cause nucleotide substitutions or deletions (De Giorgi et al., 1994). Obtaining high quality DNA from FFPE samples is limited by the duration of fixation and the length of storage time (Crisan and Mattson, 1993). Over time, formalin acidifies into formic acid further deteriorating the DNA (Ferrer et al., 2007). The DNA extraction method also limits the size of DNA fragments. It has been shown that the phenol-chloroform extraction method further damages the fragile formalin-fixed DNA (Wang et al., 1994). The use of commercial DNA extraction kits has been shown to yield higher quality DNA (Ferrer et al., 2007; Jacobs et al., 2007). Since this study used formalin fixative and phenol-chloroform extraction method, the limited number of samples (n=5) with an appropriate call rate is understandable.

The high number of Thoroughbreds in the sample set diagnosed with EPM may be misleading. Thoroughbreds may not be more susceptible to EPM than other breeds, as the data analysis needs to consider the demographics of Lexington, Kentucky from which the sample set was pulled. Due to the demographics of the horses used in the study Thoroughbred horses were overrepresented. To be conclusive, the sample set would need to contain horses from other geographic areas. For the purpose of this study, however, Thoroughbred horses were desired for the SNP analysis, as they have less diverse genetic markers than other breeds.

Based on our findings, the use of FFPE tissues for GWAS might be possible if samples were less than a year old. It is very likely that a GWAS of EPM susceptibility will have to be done prospectively, as there are not enough EPM cases reviewed at the UK VDL in a single year to obtain a significant sample set. However, if hair was collected during necropsy, and the definitive EPM diagnosis was obtained by the presence of *S. neurona* in the CNS, the DNA from the hair bulbs could be used. This would provide quality DNA to be run on the SNP chip. Additionally, different extraction methods could be utilized, such as a commercial DNA extraction kit, to obtain higher quality DNA. This methodology may be useful in studying more prevalent diseases that allow for adequate collection within one year.

CHAPTER THREE

Assessment of an equine protozoal myeloencephalitis model using an artificial infection method in horses

3.1. Introduction

The development of an experimental horse equine protozoal myeloencephalitis (EPM) model would enhance the understanding of the pathogenesis and improve diagnostics and treatment therapies of the disease. Several previous attempts have been made to create models using various strategies, each resulting in varied success. In one model, *Sarcocystis neurona* sporozoites collected from the intestines of opossums were given intragastrically to horses (Fenger et al., 1997). A dose dependent relationship was seen with horses given a larger inoculum, 10^6 sporozoites, developing more severe clinical signs (Sofaly et al., 2002). Corticosteroids (Cutler et al., 2001) and transport stress (Saville et al., 2004b; Saville et al., 2001) caused horses to seroconvert and display mild to moderate clinical signs of EPM. However, *S. neurona* were not identified in neurologic tissue histologically. In a second model, intrathecal placement of *S. neurona* merozoites caused horses to develop antibodies in the blood and CSF, but not develop clinical neurologic EPM signs (Lindsay et al., 2000).

An EPM model by Ellison et al. (2004) incubated lymphocytes with *S. neurona* merozoites overnight before intravenously injecting them back into the horse. This process was repeated for 15 consecutive days. All challenged horses developed moderate neurologic disease with antibodies present in the serum and CSF. A modified process by Ellison and Witonsky (2009) incubated *S. neurona* merozoites in whole blood overnight

before injecting back into the horse. This resulted in progressive neurological signs with antibodies present in the serum and CSF.

There is limited knowledge of the horse cell-mediated immune (CMI) response to *S. neurona*. Studies, using natural and experimentally infected horses, have provided evidence that EPM horses have an increased IL-4 (Spencer et al., 2005) and suppressed IFN-γ expression in lymphocytes (Spencer et al., 2004). It is difficult to determine, however, if the development of EPM requires immunosuppression prior to infection or if *S. neurona* has the ability to suppress the horses' immune response (Tornquist et al., 2001).

The object of this study was to evaluate whether an artificial infection model described previously (Ellison and Witonsky, 2009; Ellison et al., 2004) could be replicated to produce clinical EPM. Horses were injected intravenously with autologous blood incubated with *S. neurona* merozoites. Additionally, the immune response of horses to *S. neurona* was examined during the challenge infection.

3.2. Materials and Methods

3.2.1. Experimental Animals

Six Quarter Horse yearling horses (12 – 18 months of age; 3 male, 3 female) were acquired from a commercial source. Horses were screened for antibodies to *S. neurona* surface antigens SnSAG2 and SnSAG4/3 (Equine Diagnostic Solutions, Lexington, Kentucky). One horse (464) had an antibody titer of 1:250 and was removed from

consideration as the uninoculated control. The uninoculated control was randomly selected from the remaining group of 5 horses, all of which had an antibody titer for SnSAG2 and SnSAG4/3 of <1:250. Neurologic examinations were performed to ensure that all horses were neurologically normal prior to challenge/inoculation.

Horses were housed at East Tennessee Clinical Research, Inc. (ETCR) (Rockwood, Tennessee) in individual 12 x 12 ft. stalls within a wooden-trussed, metal-roofed building. Stalls consisted of 2 metal panels and 2 solid wooden walls, bedded with hardwood shavings and sawdust over packed clay and limestone. Horses were fed hay and grain concentrates daily in amounts adequate for proper growth and maintenance. Water was provided *ad libitum*. Horses were acclimated to study conditions for 2 days prior to first inoculation. Handling of horses was in compliance with local regulations, facility standard operating procedures, and the facility Institutional Animal Care and Use Committee (IACUC). Daily health observations of each horse were conducted by staff at ETCR, paying attention to general appearance, behavior, appetite, fecal consistency, and particularly the horses' neurologic function.

3.2.2. Experimental Infection of Horses

Sarcocystis neurona merozoites were cultured at the University of Kentucky, as described previously (Howe et al., 2005). Cultured parasites were harvested and transported at 37°C in a styrofoam container for approximately two hours to a meeting location in Jellico, Tennessee. At ETCR, 5mL peripheral blood was collected from the jugular vein of each horse into EDTA vacutainer tubes (BD Vacutainer, Franklin Lakes,

New Jersey). Blood tubes were car transported in an incubator at 37°C for two hours to the meeting location in Jellico, Tennessee. Each blood tube was inoculated with a million (1×10^6) merozoites consisting of equal parts *S. neurona* strain SN3 (Granstrom et al., 1992) and strain SN4 (Davis et al., 1991a). The control horse, 465, received only cell culture medium. The blood tubes were incubated at 37°C while traveling back to ETCR. Upon arrival, tubes were incubated at 37°C on a rocking platform for an additional two hours. Finally, autologous blood cells were injected back into each horse via intravenous injection of the jugular vein. Inoculations occurred on Days 0, 5, 12, and 20 (Figure 3.1).

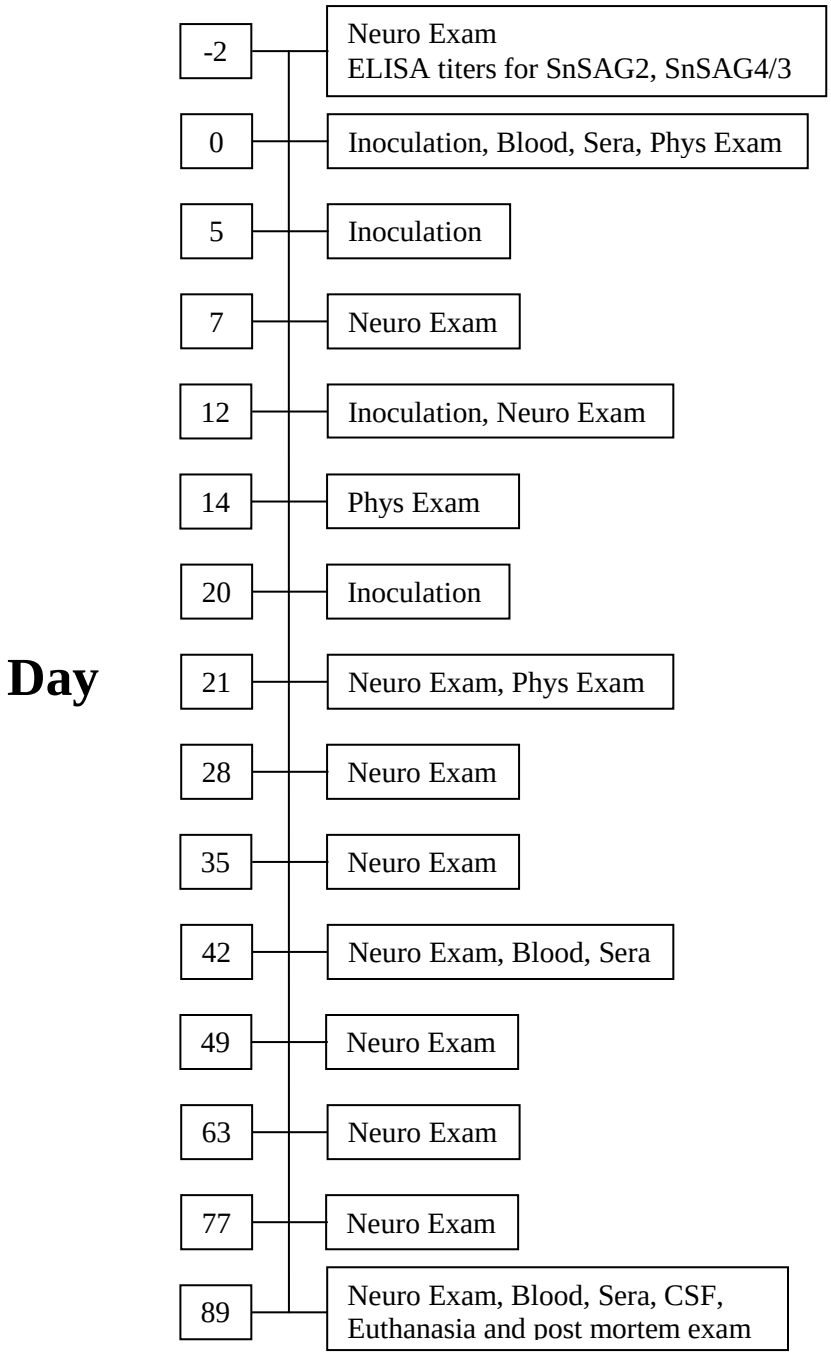

Figure 3.1. Timeline for inoculation, sample collection, and examination of horses. Clinical health observations performed daily beginning Day -2 and continuing throughout the entire study. Neuro Exam: neurologic examination; Sera: serum collected; Blood: heparinized blood collected; CSF: cerebrospinal fluid collection; Phys Exam: physical examination.

3.2.3. Sample Collection and Preparation

The timeline for sample collections is summarized in Figure 3.1. Serum samples were collected and sent to Equine Diagnostic Solutions on Day -2 to test for antibody titers against *S. neurona*. Additional serum samples were obtained on Day 0, 42, and 89 by collecting peripheral blood from the jugular vein in a 6mL serum separator vacutainer tube (BD Vacutainer). Serum tubes were allowed to clot for at least 30 minutes and then centrifuged at 3,000g for 10 minutes. Peripheral blood samples were collected from the jugular vein in two 4mL sodium heparinized vacutainer tubes (BD Vacutainer) on Day 0, 42, and 89. Immediately following euthanasia on Day 89, 3mL CSF was collected from the foramen magnum of the atlanto-occipital joint in 4mL red top tubes (BD Vacutainer) using aseptic technique.

Staff members of the ETCR performed all sample collections. Tubes were transported by car at 37°C to the University of Kentucky. Serum was removed from the tubes. Serum and CSF were stored at 4°C until assayed. Heparinized blood was immediately stimulated as described in section 3.2.9.

3.2.4. Examinations

Physical examinations were performed on Days 0, 14 and 21 by ETCR staff. Neurologic examinations were completed on Days -2, 7, 12, 21, 28, 35, 42, 49, 63, 77, and 89. Exams were conducted by Dr. Steve Reed (Rood and Riddle Equine Hospital, Lexington, Kentucky) on days -2, 12, and 42. Dr. Julio Prado (ETCR) performed the remaining neurologic examinations. The examination schedule is summarized in Figure 3.1.

Neurologic examinations evaluated each of the horses' limbs (left front, right front, left hind, and right hind) at the walk, trot, circling, and backing. Each movement was assessed for ataxia (incoordination), paresis (weakness), spasticity (stiffness), and dysmetria (range of movement), and assigned a grade of 0-5 (Table 3.1), resulting in a total of 64 scores per horse (Table 3.2).

Table 3.1. Description of ataxia grades. (Rose, 2000)

Grade	Description
0	No deficits; normal
1	Just detected at normal gait
2	Deficit easily detected and exaggerated by backing, turning, swaying, loin pressure or neck extension
3	Deficit very prominent on walking, turning, loin pressure or neck extension
4	Stumbling, tripping, falling down spontaneously
5	Recumbent, unable to rise

Table 3.2. Neurology examination score sheet. At the walk, trot, circling, and backing, horses received a score of 0-5 for each limb based on ataxia, paresis, spasticity, and dysmetria.

	Walk				Trot				Circling				Backing			
	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR	LF	LR	RF	RR
Ataxia																
Paresis																
Spasticity																
Dysmetria																

LF: left front; LR: left rear; RF: right front; RR: right rear

3.2.5. Euthanasia and Necropsy

All six horses were euthanized and necropsied on Day 89. Horses were sedated intravenously with xylazine and then injected intravenously with a lethal dose of sodium pentobarbital. The euthanasia method was consistent with specification of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia (2007). During necropsy, sections of the brain and spinal cord were collected. Specifically, the dura mater was opened and 0.5cm sections of the spinal cord were removed from each of the following sections: Cervical$(C)_{1\text{-}2}$, $C_{3\text{-}4}$, C_7-Thoracic$(T)_1$, $T_{4\text{-}5}$, $T_{8\text{-}9}$, $T_{12\text{-}13}$, Lumbar$(L)_{1\text{-}2}$, $L_{3\text{-}4}$, L_7-Sacral$(S)_2$. Tissue samples were placed in containers of buffered neutral formalin, and later sectioned, mounted, and stained for histological evaluation. Euthanasia and necropsies were performed by staff at ETCR.

3.2.6. Histological Examination

Histological examination was performed by board-eligible veterinary pathologist Dr. Uneeda Bryant at the University of Kentucky Veterinary Diagnostic Laboratory (UK VDL). Tissue sections were stained with H&E and evaluated under light microscopy for abnormalities.

3.2.7. Immunohistochemistry

Immunohistochemical (IHC) staining was completed using an automated staining system (Bond-maX, Leica Biosystems, Newcastle Upon Tyne, UK) following the manufacturer's IHC Protocol F and the Bond Polymer Refine detection system (Leica Biosystems). This

procedure involved an automated dewaxing and rehydration of the tissue, continuing with a heat-induced antigen retrieval using a ready-to-use citrate based (pH 6.0) buffer and surfactant (Leica Biosystems) at 100°C for 20 minutes. Slides were then incubated with 3% hydrogen peroxide (H_2O_2) for 5 minutes, followed by application of anti-*S. neurona* rabbit serum diluted 1:2500 with Bond Primary Antibody Diluent (Leica Biosystems), and incubated for 15 minutes. Slides were treated with postprimary blocking reagent for 8 minutes and then a horseradish peroxidase-labeled IgG polymer for 8 minutes. Diaminobenzidine tetrahydrochloride (DAB) substrate was added, and the slides were incubated for 10 minutes. Finally, the slides were counterstained with hematoxylin for 5 minutes. Between each incubation step, slides were washed using Bond Wash Solution 10x Concentrate (Leica Biosystems), diluted with distilled water to a 1x working concentration, to remove any unbound material. Brain tissue from a clinical EPM horse with a large number of *S. neurona* organisms was stained as a control. A negative control consisted of only Bond Primary Antibody Diluent (Leica Biosystems).

Slides were removed from the instrument and washed under distilled water. Tissue was dehydrated using a graded series of alcohol washes (2-85% ethanol, 2-90% ethanol, 2-95% ethanol, 2-100% ethanol) and cleared with 4 xylene washes. Slides were covered using mounting medium and glass coverslips, and viewed with light microscopy to identify *S. neurona* organisms.

3.2.8. Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) were conducted essentially as described previously (Hoane et al., 2005; Yeargan and Howe, 2011). Each rSnSAG was diluted in phosphate buffered saline (PBS) at predetermined optimal concentrations (SnSAG1 0.2µg/mL, SnSAG2 1.0µg/mL, SnSAG4/3 0.5µg/mL, SnSAG5 0.5µg/mL), and then 75µL of the antigen was dispensed into each well of high binding 96-well plates (Corning, Corning, New York). Plates were incubated overnight at 4°C. Antigen was removed and plates rinsed three times using PBS with 0.05% Tween 20 (PBST). Wells were blocked with 200µL blocking solution (PBS containing 5% normal goat serum, 1% Tween 20, 1% nonfat dry milk powder) and incubated at room temperature (RT) for 1.5 hours. Wells were rinsed a single time with PBST. Primary antibody was diluted (serum 1:250, CSF 1:2.5) with antibody diluent solution (1:10 blocking solution with PBST), 75µL was added to duplicate wells, and incubated at 37°C for 1 hour. Plates were washed 5 times with PBST. Secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-horse immunoglobulin (Ig)G (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania) diluted 1:10,000 in antibody diluent, was added to wells in 75µL aliquots and incubated at 37°C for 1 hour. The plates were washed 5 times with PBST, then 75µL of RT chromagenic substrate TMB (3,3',5,5'-tetramethylbenzidine; Pierce, Thermo Fisher Scientific, Inc., Waltham, Massachusetts) was added to each well. Plates sat at RT for 20-30 minutes for color to develop. Reactions were stopped with 75µL of 2M sulfuric acid. Optical density (OD) was measured at 450nm (OD_{450}) in an E_{max} microplate reader (Molecular Devices, Sunnyvale, California).

To account for inter-plate variability, percent positivity values were calculated for each sample relative to the reference standards [(OD(sample) – OD(negative control)) / (OD(positive control) – OD(negative control))] X 100 (Wright et al., 1993). The positive controls were serum samples from symptomatic horses with histologically-confirmed EPM. The negative control was a serum sample from a pre-infection weanling used in a previous *S. neurona* challenge study (Fenger et al., 1997). At a dilution of 1:250 (serum) or 1:2.5 (CSF), a sample is positive if the percent positivity is above the cutoff value of 15% for SnSAG1, SnSAG2 and SnSAG5, and 10% for SnSAG4/3 (Hoane et al., 2005; Yeargan and Howe, 2011). End-point titers, the last dilution with a percent positivity above a set cutoff value, were established with serial 2-fold dilutions of serum and CSF samples beginning with 1:250 (serum) or 1:2.5 (CSF). A positive serum sample has at least an end-point titer of 1:250. Samples below the cutoff value at a 1:250 dilution are negative and given the antibody titer of <1:250.

ELISAs for IgG isotypes were essentially conducted as stated above, except utilizing different secondary antibodies. Monoclonal antibodies (mAb) specific for IgGa (CVS 48; 1:100), IgGb (CVS39; 1:10), and IgG(T) (CVS40; 1:10) (Lunn et al., 1991) were diluted to optimal concentrations with antibody diluent solution. Wells were incubated 1 hour at 37°C with 75µL mAb diluent solution. Plates were rinsed 5 times with PBST, and incubated 1 hour at 37°C with 75µL of HRP-goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc.) diluted 1:10,000 in antibody diluent solution.

3.2.9. Relative Quantification (RQ) of Cytokine mRNA Expression by Real-time PCR

Upon arrival at the University of Kentucky on Days 0, 42, and 89, paired heparinized blood tubes were measured to ensure each tube contained 2.5mL of blood. A 10^8 pellet of frozen SN3 *S. neurona* merozoites was resuspended in 1mL of RPMI 1640 medium (HyClone, Thermo Fisher Scientific, Inc.), and 100µL of the suspension was added to one blood tube from each of the six horses. Blood tubes were incubated at 37°C for 24 hours. Each blood tube was pipetted into a PAXGENE blood RNA tube (PreAnalytiX, Valencia, California), incubated at RT for 24 hours, and then stored at -20°C.

Total RNA was extracted using the PAXGENE blood RNA extraction kit (Qiagen, Valencia, California) following manufacturer's protocol. PAXGENE tubes (PreAnalytiX) were thawed, mixed thoroughly, and centrifuged 10 minutes at 2,800g. The supernatant was poured off, and pellets were resuspended in 4mL of RNAse-free water (Qiagen). Tubes were centrifuged for 10 minutes at 2,800g. The supernatant was discarded. Pellets were resuspended in 350µL Buffer BR1 (Qiagen) and transferred to 1.5mL Eppendorf tubes. To each tube, 300µL Buffer BR2 (Qiagen) and 80µL Proteinase K (Qiagen) was added, with samples incubated 5 minutes at RT, then 10 minutes at 55°C. Tubes were thoroughly mixed and centrifuged 10 minutes at 20,000g. Pellets were removed and discarded. The supernatants were mixed with 350µL of 100% ethanol. Spin columns, placed in 2mL collection tubes, were centrifuged 1 minute at 8,000g with 700µL of sample. After each centrifugation, the spin columns were transferred to new 2mL collection tubes, and filled with the remaining sample volume, and centrifuged 1 minute at 8,000g. This process was repeated three times for 1 minute at 8,000g using 700µL

Buffer BR3 (Qiagen), for 1 minute at 8,000g using 500µL Buffer BR4 (Qiagen), and for 3 minutes at 20,000g using 500µL Buffer BR4 (Qiagen). Spin columns were then centrifuged again for 1 minute at 20,000g with no buffer added. Spin columns were placed in 1.5mL Eppendorf tubes with 40 µL Buffer BR5 (Qiagen) added to the columns, and centrifuged for 1 minute at 8,000g. The process was repeated a second time. Spin columns were discarded after second centrifugation with Buffer BR5 (Qiagen). The tubes were incubated 5 minutes at 65°C, then immediately placed on ice. The RNA concentration was quantified at OD_{260} using an Eppendorf Biophotometer (Hauppauge, New York).

In PCR tubes, 0.5µg of each RNA sample was brought up to a volume of 41.5µL with RNAse-free water (Qiagen), and mixed with 38.5µL of reverse transcription master mix. Reverse transcription master mix contained 0.5µL avian myelobastosis virus (AMV) reverse transcriptase (20U/µL; Promega, Madison, Wisconsin), 1µL oligo dT primer (0.5µg/µL; Promega), 1µL RNAsin (40U/µL; Promega), 4µL dNTP (10mM; Promega), 16µL $MgCl_2$ (25mM; Promega), and 16µL AMV buffer 5x (Promega). Reactions were incubated at 42°C for 15 minutes, then 95°C for 5 minutes in a thermocycler. The cDNA samples were stored at -20°C until further analysis.

Gene expression utilized equine specific intron-spanning beta-glucuronidase (β-GUS), Granzyme B (GrzB), IFN-γ, IL-2, IL-4, and T-bet primer/probe sets (Applied Biosystems, Foster City, California) (Table 3.3). The cDNA was diluted 1:1 with RNAse-free water (Qiagen), and reactions were setup using an automated PCR setup machine (Corbett). Each reaction consisted of 4.5µL diluted cDNA, 5µL TaqMan® Gene

Expression Master Mix (Applied Biosystems), and 0.5µL primer/probe set (Applied Biosystems). Samples were run in duplicate using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Reactions were incubated for 10 minutes at 95°C, and then underwent 40 cycles of 95°C for 15 seconds, followed by 1 minute at 65°C. The PCR efficiencies were determined using LinRegPCR (Ramakers et al., 2003). Gene expression levels were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), where $\Delta\Delta C_T = [(\text{Mean gene of interest } C_T - \text{Mean } \beta\text{-GUS } C_T)_{Horse} - (\text{Mean gene of interest } C_T - \text{Mean } \beta\text{-GUS } C_T)_{Calibrator}]$. The calibrator for each horse was the non-stimulated media control of the pre-challenge sample. The reference gene β-GUS was used as an endogenous control (Breathnach et al., 2006). Results were calculated as $2^{-\Delta\Delta CT}$ and denoted as relative quantification (RQ), measuring fold changes of expression levels.

Table 3.3. Primer probe sets used for Real-time PCR.

Gene	Reference
β-GUS	Liu et al., 2011
GrzB	Liu et al., 2011
IFN-γ	Adams et al., 2011
IL-2	Adams et al., 2011
IL-4	Adams et al., 2011
T-bet	Ainsworth et al., 2003

3.2.10. Statistics

Statistical analyses were run on SIGMASTAT™ (Systat Inc. Richmond, California). Two-way repeated measured analysis of variance (ANOVA) was used to detect significant differences between the gene expression and the ELISA end-point titers, and

IgG isotype percent positivity values for the three sample days of the challenged horses. Data was $\log_{10}$ transformed, if not distributed normally, prior to analysis. Differences were considered to be significant at $P < 0.05$. For the CMI assay, comparisons were made from the mean difference between *S. neurona* stimulated and non-stimulated blood at different time points for the challenged horses. As there was only a single control horse, statistical comparisons could not be made between the challenged and control groups.

3.2.11. Sarcocystis neurona Merozoite Invasion Assay

A confluent T25 flask of bovine turbinate (BT) cells was washed twice with 37°C PBS. Cells were incubated with 0.5mL warm trypsin-versene solution. Once cells sloughed off, they were resuspended in 15mL 4% FBS RPMI 1640, and aliquotted 0.5mL into each well of a 24-well plate. Plate was incubated at 37°C for 5 days.

As previously described (Howe et al., 2005), *S. neurona* strain F9F merozoites that express yellow fluorescent protein (YFP) were propagated by serial passages in BT cells. Merozoites were harvested from infected BT monolayer cultures that had fully lysed. Merozoites were pelleted by centrifugation at 3,000g for 10 minutes, then resuspended in 4mL 4% FBS RPMI 1640. Parasites were passed through 22 and 25 gauge needles, and then through a 3.0µm pore sized membrane filter. The merozoite suspension was placed in a styrofoam container filled with 37°C water bottles to replicate conditions during the car transport. The styrofoam container was kept at room temperature. The BT cell monolayer in the 24-well plate received fresh media, 0.5mL 4% FBS RPMI 1640. At different time points (0, 1, 2, 3, 4, 6 hours), 20µL of the *S. neurona* merozoite suspension

(2,000 parasites) was added to three wells. The plate was kept at 37°C for 4 days. The YFP-expressing parasites were counted using a Nikon Diaphot inverted microscope equipped for fluorescence microscopy.

3.3. Results

3.3.1. Clinical Findings

Neurological examinations prior to inoculation (Day -2) were within normal limits for all horses (Figure 3.2). Horses 466, 467, 468 and the control began showing detectable neurological abnormalities (score >1) on day 7 post inoculation (PI), and horses 463 and 464 began showing abnormalities on day 12. The highest score received by horses 464 and the control was 2. Scores of 3 were received by horse 468 on day 42, by horses 463 and 467 on day 63, and by horse 466 on day 89. There were no scores of 4 or 5 given to any horse during the study. Physical examinations and daily clinical observations did not detect any non-neurologic abnormalities.

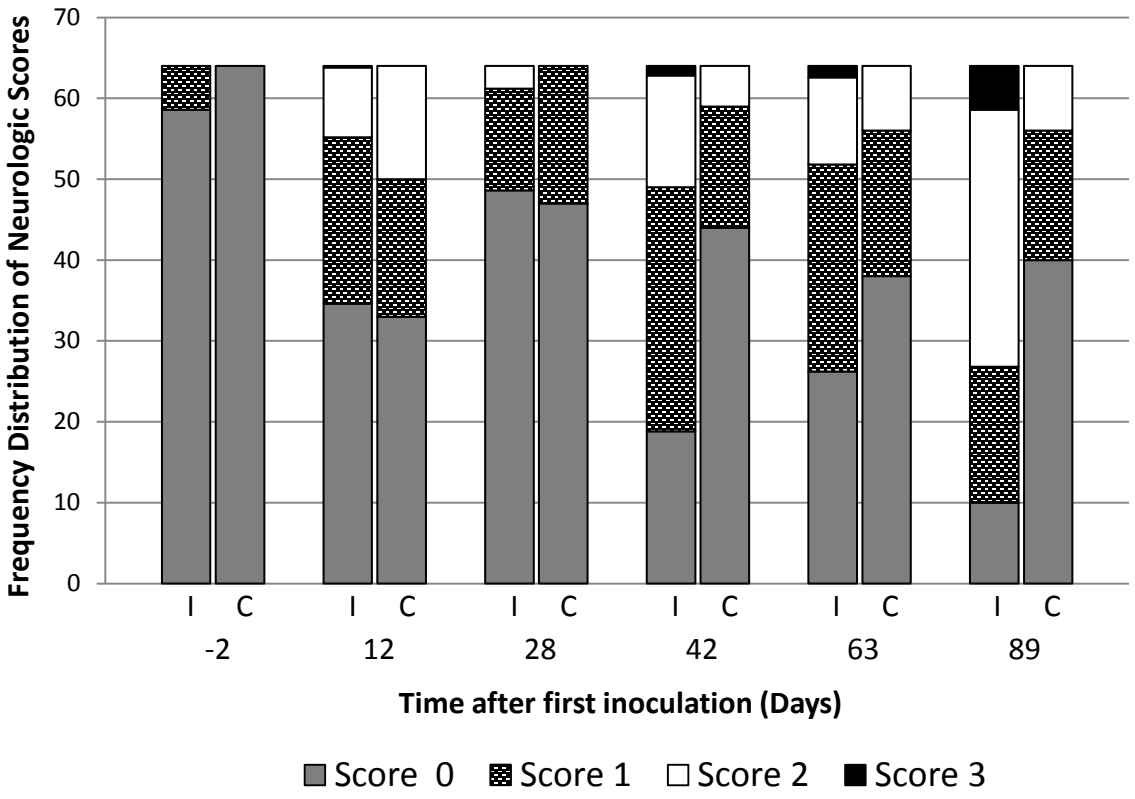

Figure 3.2. Neurology examination scores. For each neurologic examination, horses received 64 scores of 0-5. All horses were neurologically normal at the beginning of the study. Progressively, horses developed increasing neurological deficits. Scores represent the average scores of the challenged group, with the raw scores of the control horse. I: Inoculated horses; C: Control horse.

3.3.2. Postmortem Examination

Minimal histopathologic abnormalities were observed. Focal gliosis was apparent in the lumbar tissue of the control horse (Figure 3.3), horse 467 (Figure 3.4) and horse 468 (Figure 3.5). Additionally, horse 468 had leukocytosis (increased numbers of intravascular neutrophils and eosinophils) of a meningal vessel and a blood vessel within

the white matter. Protozoa were not identified in any tissue sections. None of the histopathological findings were consistent with protozoal infection.

Immunohistochemistry was performed on tissue sections with any abnormalities observed with H&E staining for potential identification of *S. neurona* organisms. Three tissue sections were stained, including lumbar tissue of horses 467, 468, and the control. No *S. neurona* organisms were observed in the tissues.

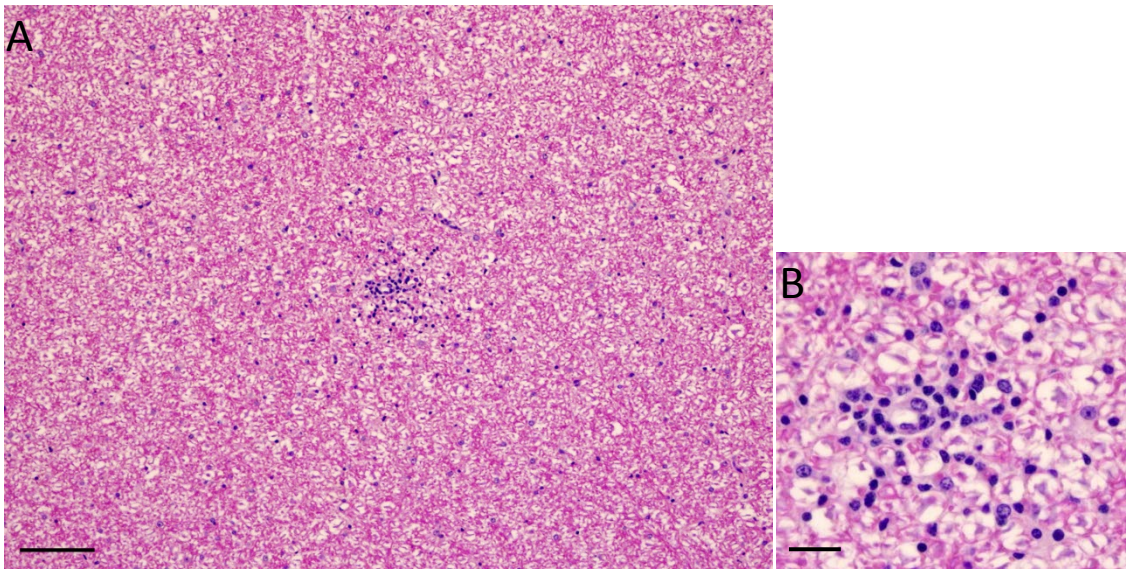

Figure 3.3. Histological evaluation of the control horse. Hematoxylin and eosin stain.

(A) Focal gliosis located within the lumbar region of spinal cord. Bar = 100µm.

(B) Higher magnification of focal gliosis in Figure 3.3A. Bar = 20µm.

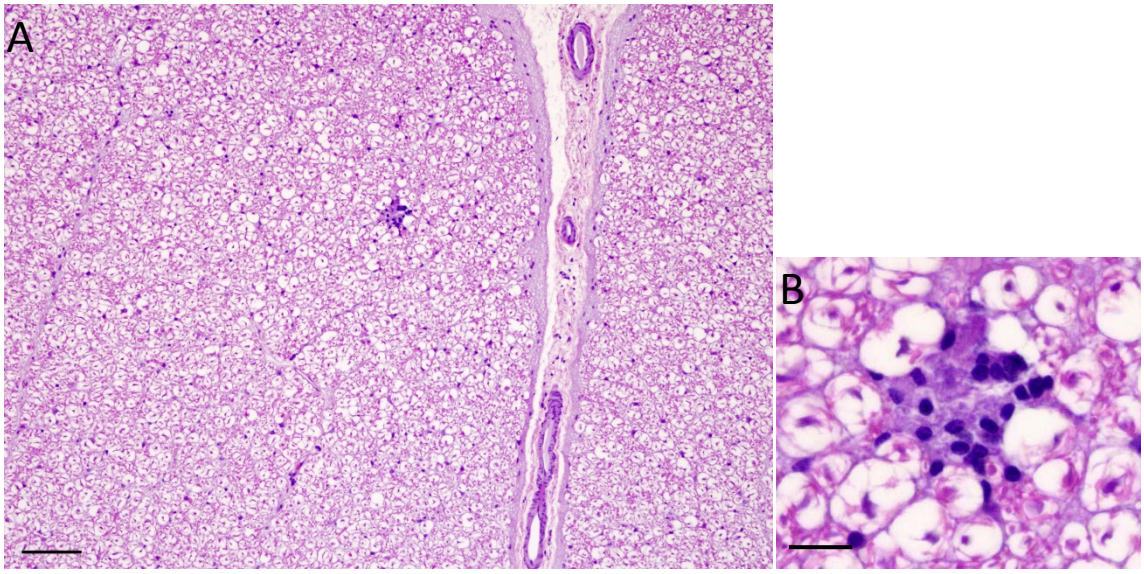

Figure 3.4. Histological evaluation of Horse 467. Hematoxylin and eosin stain.

(A) Focal gliosis located within the lumbar region of the spinal cord. Bar = 100µm.

(B) Higher magnification of focal gliosis in Figure 3.4A. Bar = 20µm.

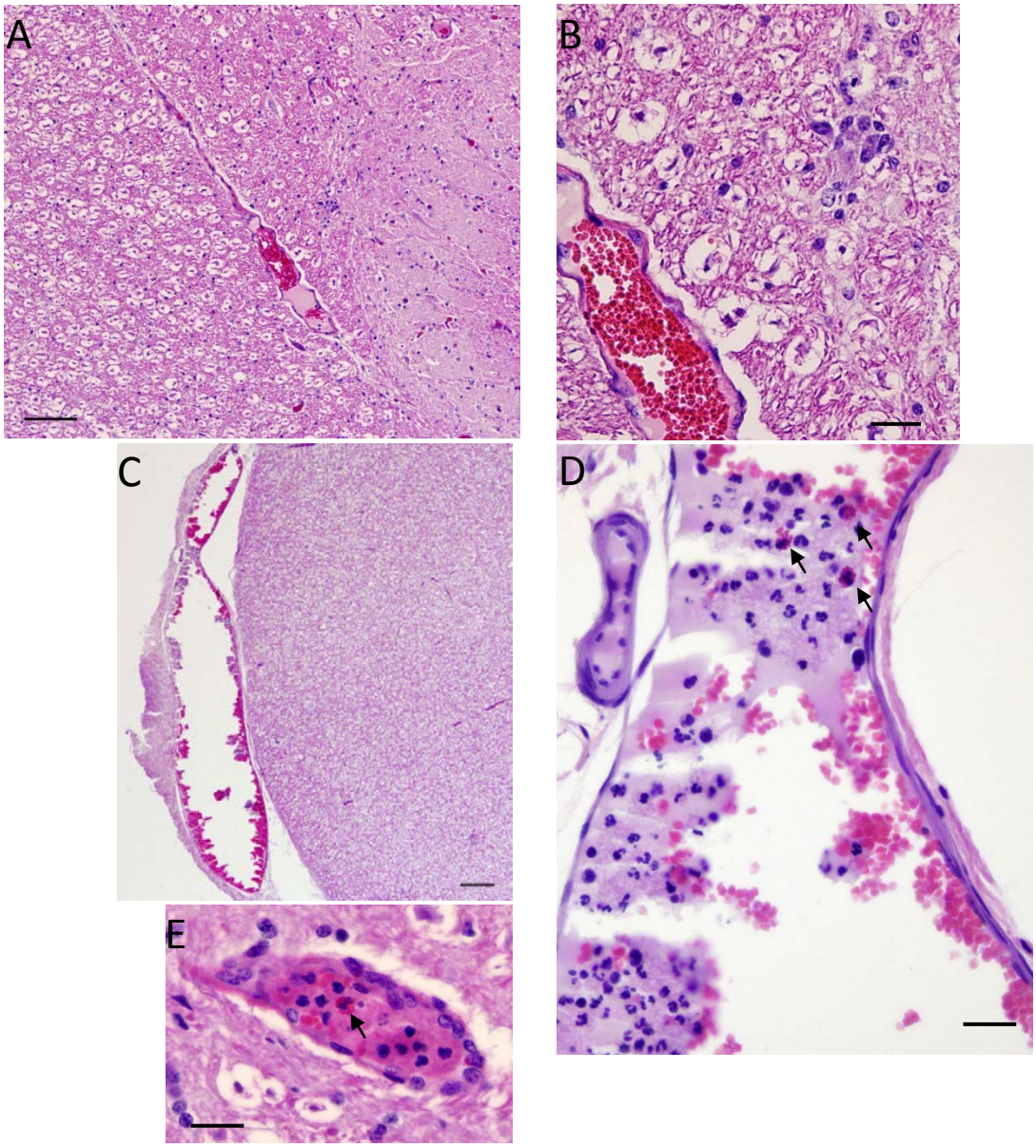

Figure 3.5. Histological evaluation of Horse 468. Hematoxylin and eosin stain. (A) Focal gliosis located within the lumbar region of the spinal cord. Bar = 100µm. (B) Higher magnification of focal gliosis in Figure 3.5A. Bar = 20µm. (C) Leukocytosis of meningal vessel. Bar = 100µm. (D) Higher magnification of Figure 3.5C. Leucocytosis of the meningal vessel with neutrophils and eosinophils (arrows) present. Bar = 20µm. (E) Leukocytosis of blood vessel within the white matter of the lumbar spinal cord with neutrophils and eosinophils (arrow) present. Bar = 20µm.

3.3.3. Antibody Response

3.3.3.1. Total IgG in Serum

Serum (pre-challenge, days 42 and 89) from all six horses was analyzed by ELISA to assess the total IgG antibody response against the parasite surface antigens SnSAG1, SnSAG2, SnSAG3, SnSAG4, and SnSAG5. End-point antibody titers were determined on all serum samples as the lowest dilution at which the percent positivity was greater than the cutoff value for each SnSAG. The control horse had sera end-point titers of <1:250 on all sample days for SnSAG1, SnSAG2, SnSAG4/3 and SnSAG5 (Figure 3.6).

For SnSAG1, all challenged horses had serum end-point titers of <1:250 pre-challenge (Figure 3.6A). On day 42, all samples were positive with end-point titers of 1:1000 for horse 466, 1:500 for horses 463 and 464, and 1:250 for horses 467 and 468. On day 89, horses 463, 464, 466 remained positive with end-point titers of 1:250, while horses 467 and 468 were negative with a titer of <1:250. The SnSAG1 serum end-point titers of the challenged horses were statistically significant between the different time points ($P = 0.004$). Day 42 serum titers were statistically significant from the pre-challenge and day 89 serum titers ($P < 0.05$).

Serum end-point antibody titers for SnSAG2 were <1:250 for all challenged horses in the pre-challenge and day 89 samples (Figure 3.6B). On day 42, horses 464 and 468 had titers of 1:500, and horses 463 and 466 a titer of 1:250. Horse 467 was negative with a titer of <1:250. The SnSAG2 serum end-point titers of the challenged horses were statistically significant between the different time points ($P = 0.006$). Day 42 serum titers were statistically significant from the pre-challenge and day 89 serum titers ($P < 0.05$).

All challenge horses on all sample days had serum end-point antibody titers of <1:250 for SnSAG4/3 (Figure 3.6C).

Challenged horses all had serum end-point titers of <1:250 in the pre-challenge serum for SnSAG5 (Figure 3.6D). End-point titers on day 42 were 1:1000 for horses 463 and 464, 1:500 for horse 466, 1:250 for horse 467, and <1:250 for horse 468. Day 89 antibody titers were 1:250 for horses 463 and 464, and <1:250 for horses 466, 467, and 468. The SnSAG5 serum end-point titers of the challenged horses were statistically significant between the different time points ($P = 0.013$). Day 42 serum titers were statistically significant from the pre-challenge and day 89 serum titers ($P < 0.05$).

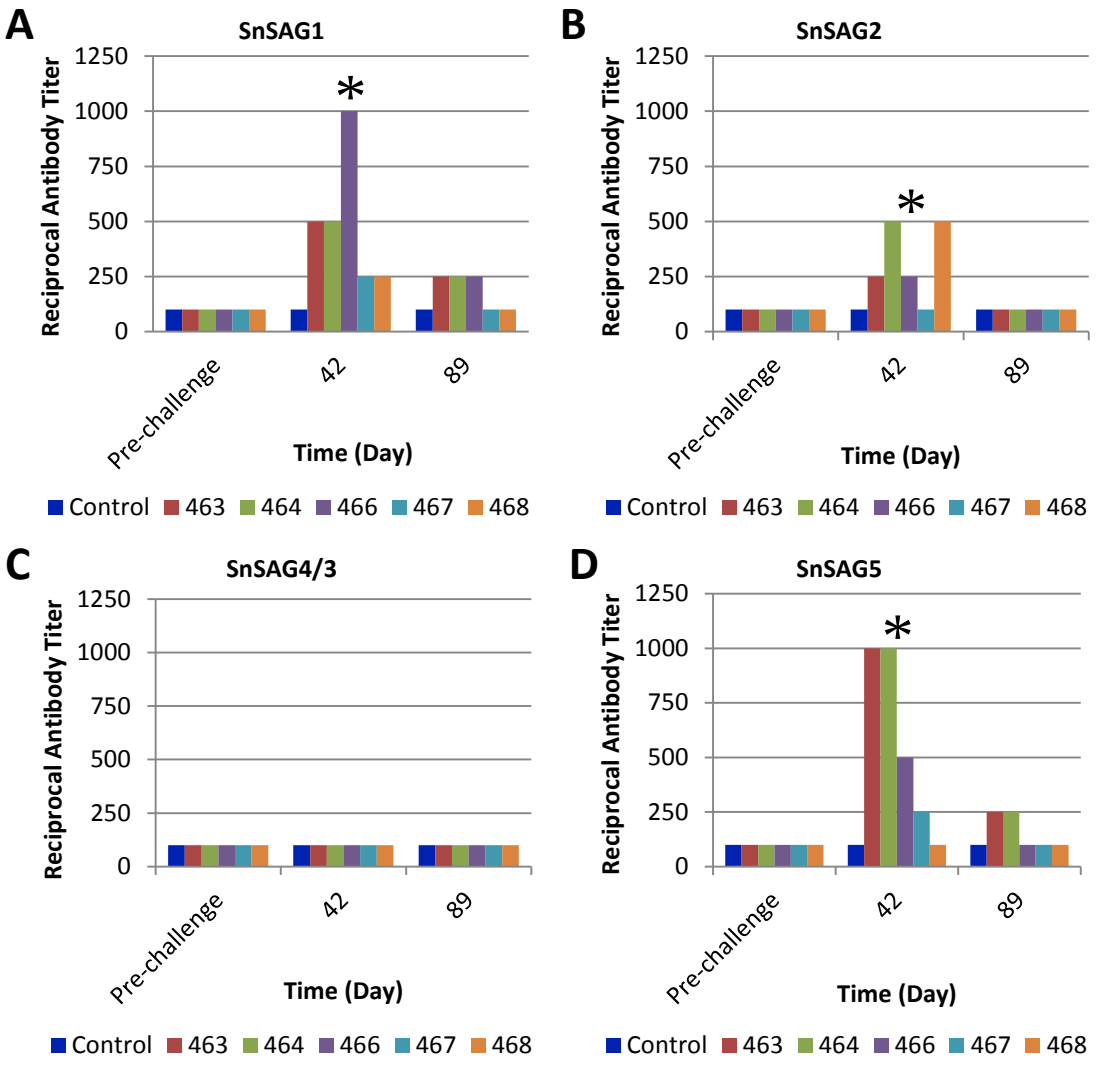

Figure 3.6. Anti-*Sarcocystis neurona* total IgG antibody titers of sera by ELISA analysis. Serum samples were collected pre-challenge and on days 42 and 89 post inoculation. Serum was tested in duplicate serial dilutions beginning at 1:250. Reciprocal antibody titers were determined as the lowest dilution at which the percent positivity was greater than the cutoff value of (A) SnSAG1, (B) SnSAG2, (C) SnSAG4/3, (D) SnSAG5. A positive serum sample has an end-point titer of 1:250. Samples below the cutoff value at a 1:250 dilution were given the antibody titer of <1:250. *Statistical difference of the serum end-point titers of challenged horses between samples days ($P < 0.05$).

3.3.3.2. Total IgG in CSF

CSF collected from horses on day 89 was analyzed by ELISA to assess total IgG antibody against the surface antigens. The control horse was negative for all SnSAGs (Figure 3.7). Horse 464 was the only challenged horse with antibodies against SnSAG1 in CSF (Figure 3.7A). The CSF from all other challenged horses was negative for all SnSAGs (Figure 3.7A-D).

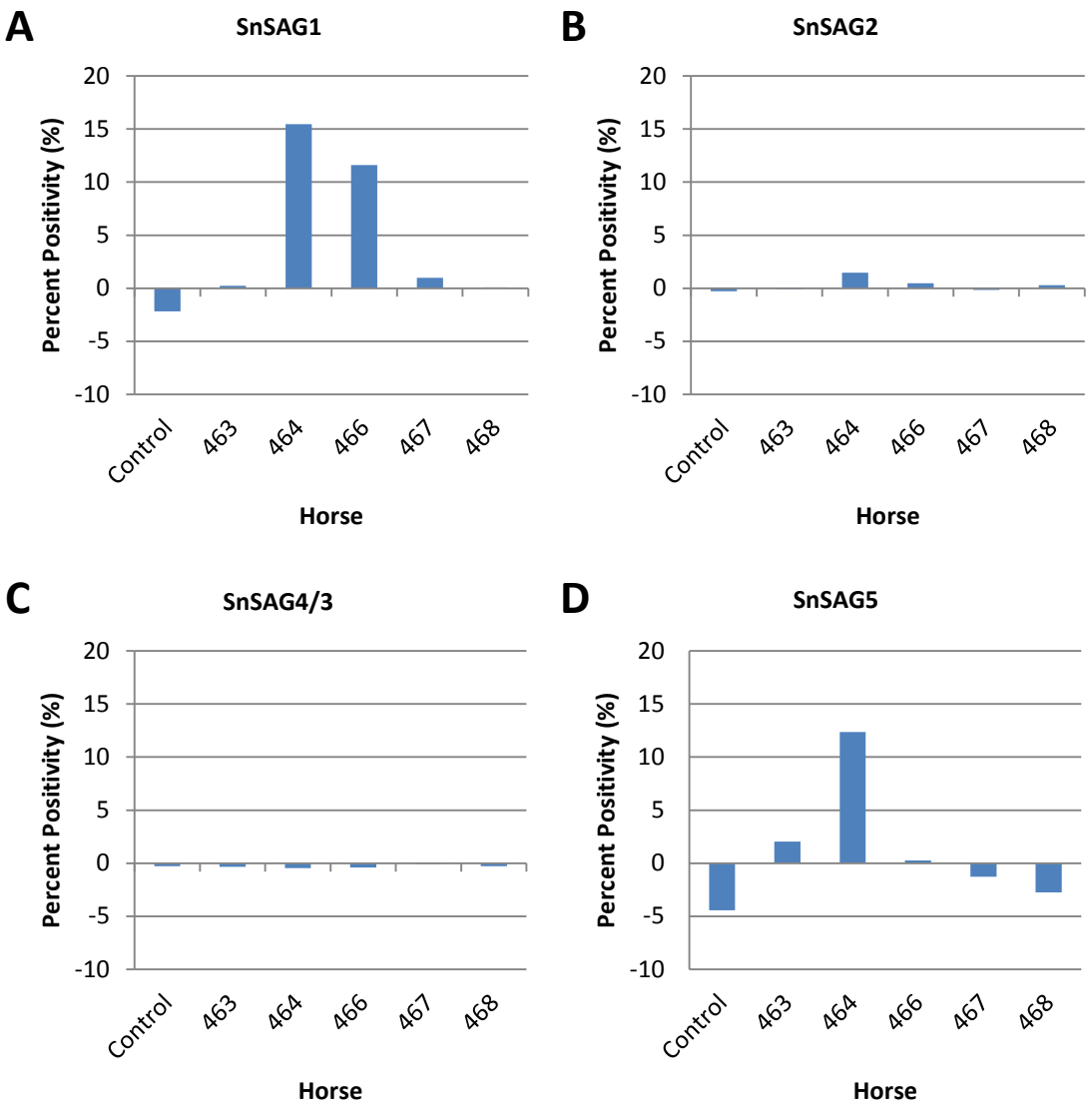

Figure 3.7. Percent positivity of anti-*Sarcocystis neurona* total IgG antibodies of CSF by ELISA analysis. CSF samples were collected on day 89. CSF was tested in duplicate at a dilution of 1:2.5 for ELISA analysis of total IgG antibodies against four SnSAGs. A positive sample has a percent positivity above the cutoff value of each SnSAG.

(A) SnSAG1 cutoff value is 15%. (B) SnSAG2 cutoff value is 15%. (C) SnSAG4/3 cutoff value is 10%. (D) SnSAG5 cutoff value is 15%. Horse 464 had a 15.4% positivity agaist SnSAG1. All other samples were negative against all SnSAGs.

3.3.3.3. IgG Isotype Response in Serum

Serum (pre-challenge, day 42 and 89) from all six horses was analyzed with ELISA, testing three different IgG isotypes (IgGa, IgGb, and IgG(T)) responses against SnSAG1 and SnSAG5.

All pre-challenge samples were negative for IgGa against SnSAG1 (Figure 3.8A). The control horse remained negative for all sample days. The entire challenged group was positive on day 42. By day 89, only horses 464, 466, and 468 were positive. The mean percent positivity of the SnSAG1 IgGa response of the challenged horses was statistically significant ($P = 0.009$) between time points. There was a significant difference between day 42 and both pre-challenge and day 89 means ($P < 0.05$).

Pre-challenge sera of all horses was negative for IgGb antibodies against SnSAG1 (Figure 3.8B). The control horse remained negative for all sample days. All challenged horses were positive on day 42 and negative on day 89. The mean percent positivity of the SnSAG1 IgGb response of the challenged group was statistically significant ($P = 0.001$) between time points. The day 42 mean was statistically significant from the pre-challenge and day 89 mean ($P < 0.05$).

Only horse 468 was positive pre-challenge for IgG(T) antibodies against SnSAG1 (Figure 3.8C). The control horse was negative for SnSAG1 IgG(T) antibodies on all sample days. All challenged horses were positive on day 42. Horse 463 was the only negative challenged horse on day 89. Between sample days there was a statistically significant difference of the mean percent positivity for the challenged group ($P = 0.008$).

The mean percent positivity of day 42 was statistically significant from both pre-challenge and day 89 means ($P < 0.05$).

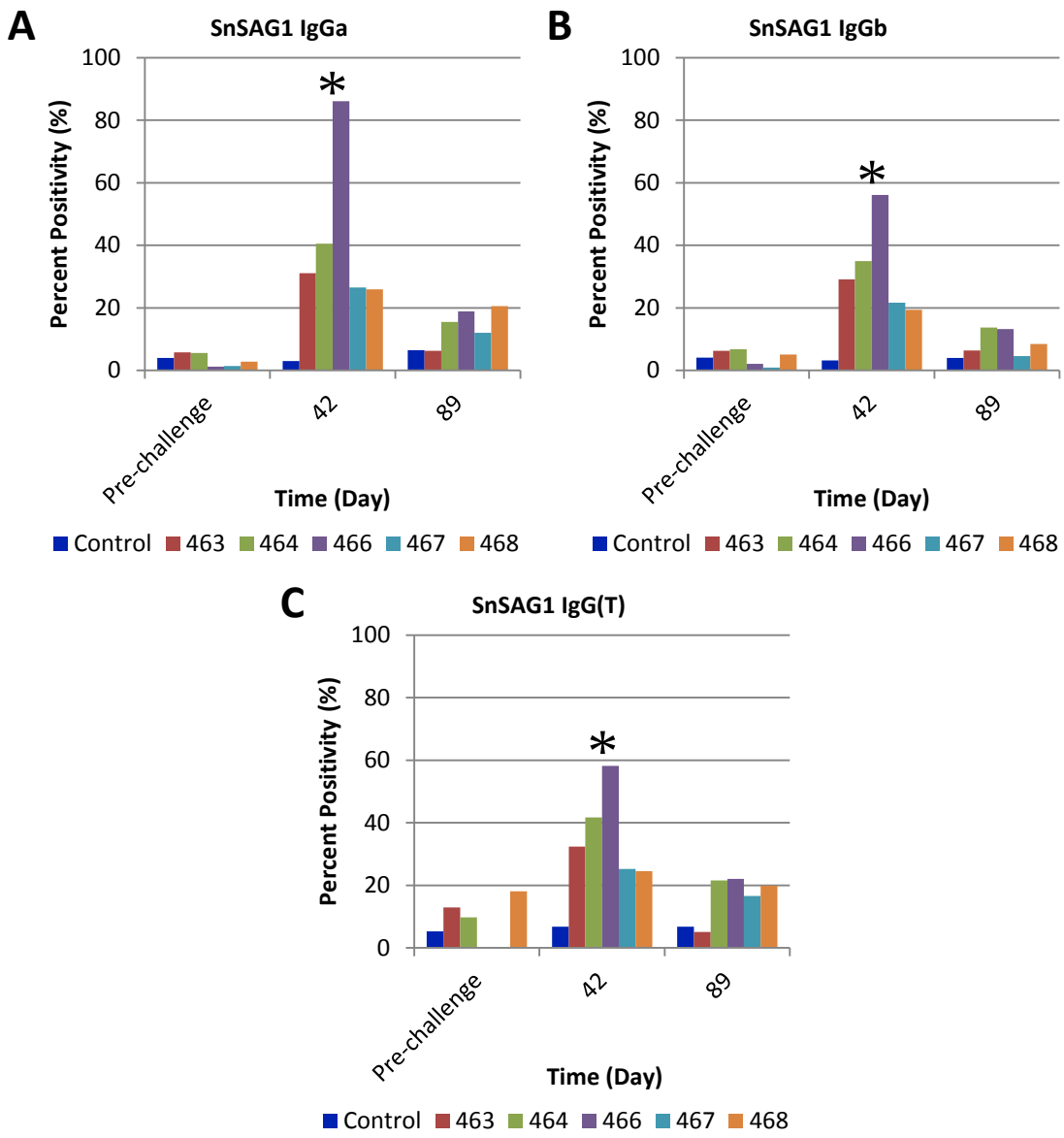

Figure 3.8. Percent positivity of anti-*Sarcocystis neurona* SAG1 IgG isotype antibodies of sera by ELISA analysis. Serum samples were collected pre-challenge and on days 42 and 89 post inoculation. Serum was tested in duplicate at dilution of 1:250 for ELISA analysis of (A) IgGa, (B) IgGb, (C) IgG(T) antibody levels against SnSAG1. A positive sample has a percent positivity above the cutoff value of 15%. The control horse was negative on all sample dates. *Statistical difference of the mean percent positivity of challenged horses between samples days ($P < 0.05$).

The control horse was positive for IgGa antibodies against SnSAG5 at pre-challenge and negative on day 42 and 89 (Figure 3.9A). Horses 463 and 466 were positive for IgGa antibodies pre-challenge. All challenged horses were positive on day 42. Only horses 467 and 468 were negative on day 89. The mean percent positivity was statistically significant between sample days for the challenged horses ($P < 0.001$). The mean percent positivity on day 42 was statistically significant from the pre-challenge and day 89 means ($P < 0.05$).

Horse 463 and the control were positive for SnSAG5 IgGb antibodies at pre-challenge (Figure 3.9B). The control horse was negative on day 42 and 89. All challenged horses were positive on day 42. Only horses 463, 464, and 466 were positive on day 89. The mean percent positivity between time points was statistically significant for the challenged group ($P < 0.001$). Day 42 mean percent positivity was statistically significant from pre-challenge and day 89 means ($P < 0.05$).

The control and horses 463 and 466 were positive for IgG(T) antibodies against SnSAG5 in the pre-challenge sample (Figure 3.9C). The control horse was negative on day 42 and 89. All challenged horses were positive on day 42. Of the challenged horses, only 466 and 467 were negative on day 89. The mean percent positivity was statistically significant between sample days ($P < 0.001$) for the challenged horses. Day 42 mean percent positivity was statistically significant from pre-challenge and day 89 means ($P < 0.05$).

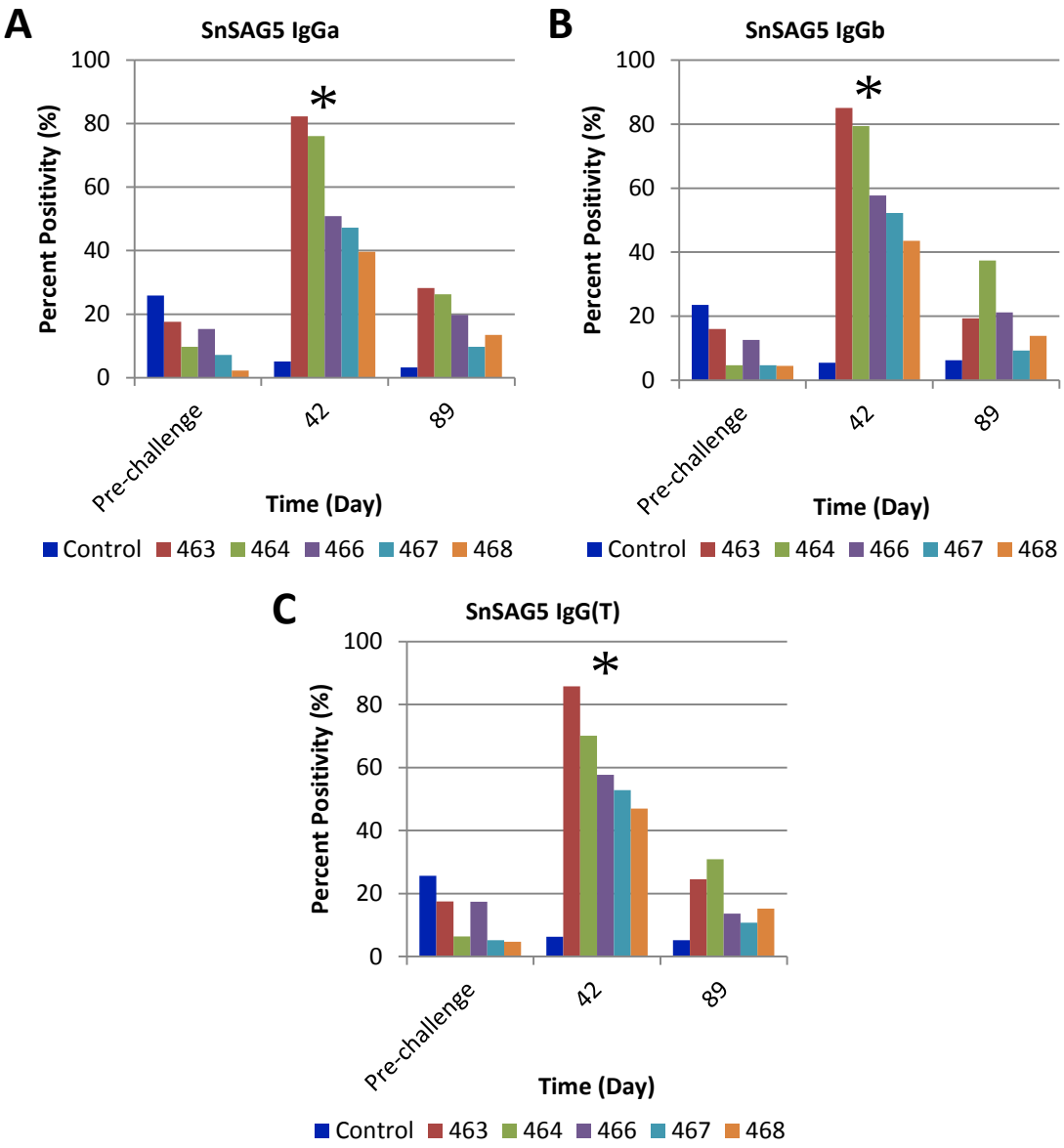

Figure 3.9. Percent positivity of anti-*Sarcocystis neurona* SAG5 IgG isotype antibodies of sera by ELISA analysis. Serum samples were collected pre-challenge and on days 42 and 89 post inoculation. Serum was tested in duplicate at dilution of 1:250 for ELISA analysis of (A) IgGa, (B) IgGb, (C) IgG(T) antibody levels against SnSAG5. A positive sample has a percent positivity above the cutoff value of 15%. The control horse had a pre-challenge reactivity over 20% for each IgG isotype. *Statistical difference of the mean percent positivity of challenged horses between samples days ($P < 0.05$).

3.3.4. Cell-mediated Immune Response

To investigate the effect of *S. neurona* on the CMI, heparinized blood samples (pre-challenge, day 42 and 89) were stimulated with SN3 strain *S. neurona* merozoites. Using real-time PCR, fold changes in mRNA expression levels are reported as RQ values.

The general expression pattern was similar for all genes in both the control and challenged horses. The fold change of expression increased on day 42. The levels declined back towards the pre-challenge baseline on day 89. The IFN-γ expression levels between sample days were significant ($P = 0.008$) (Figure 3.10A). The expression level on day 42 was significantly higher than the pre-challenge and day 89 levels ($P < 0.05$). The IL-2 expression levels were significantly different between sample days ($P = 0.042$) (Figure 3.10B). There was not enough power in the sample set to detect differences between specific time points. There were no statistically significant differences between the stimulated and non-stimulated samples among the time points for Granzyme B (Figure 3.11A), T-bet (Figure 3.11B), and IL-4 (Figure 3.12).

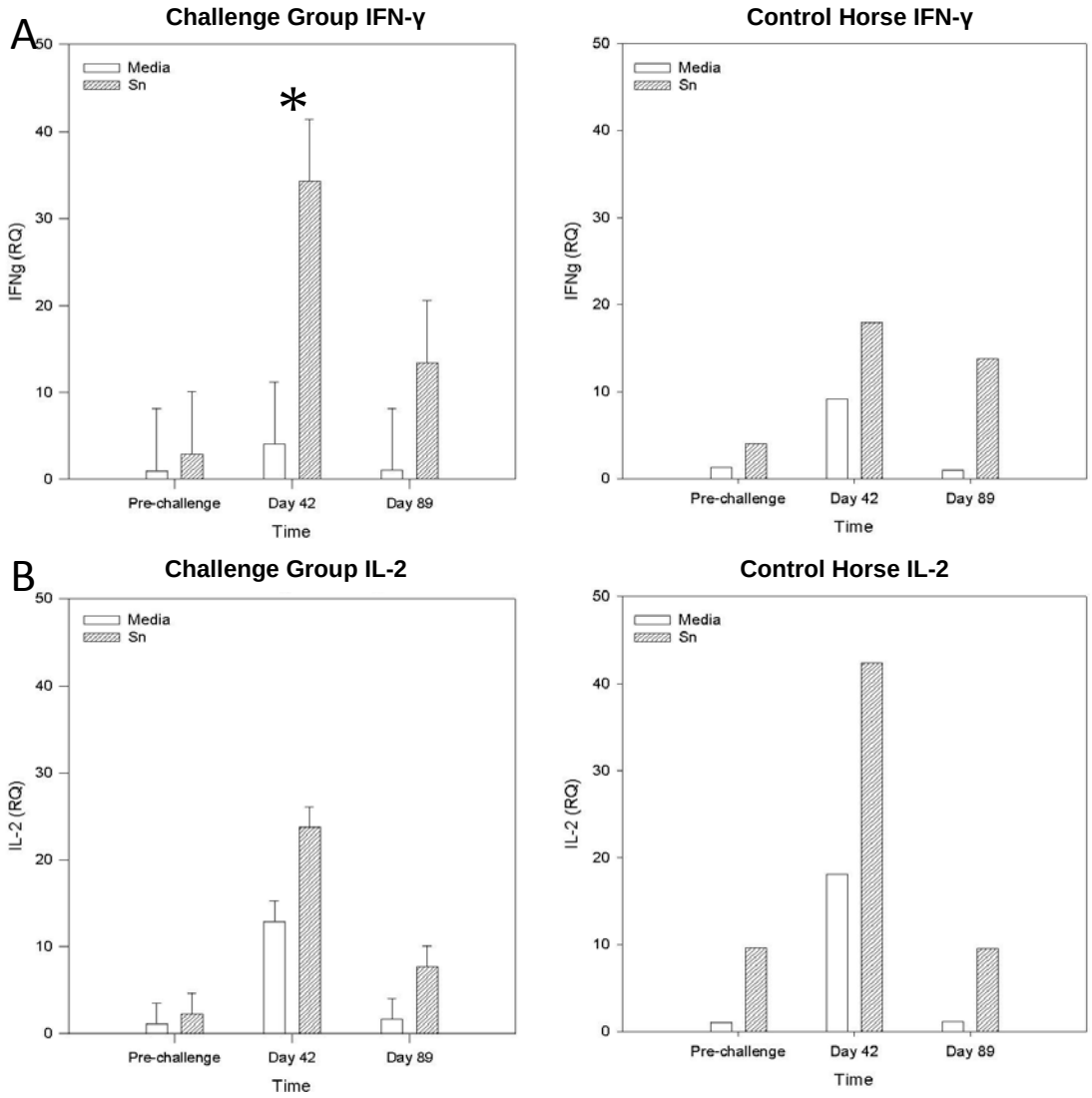

Figure 3.10. Relative quantification (RQ) of proinflammatory cytokine mRNA expression by Real-time PCR after *Sarcocystis neurona* stimulation. Heparinized blood was stimulated with SN3 strain *S. neurona* merozoites pre-challenge and on days 42 and 89 post inoculation. Mean (±SE) fold changes in expression of cytokines (A) IFN-γ and (B) IL-2 for stimulated (Sn - striped bars) and non-stimulated (Media - white bars) blood of the challenge group and control horse. Raw RQ values are presented in graphs. Data was $\log_{10}$ transformed for statistical analysis. *Difference between stimulated and non-stimulated samples was statistically significant among the time points ($P < 0.05$).

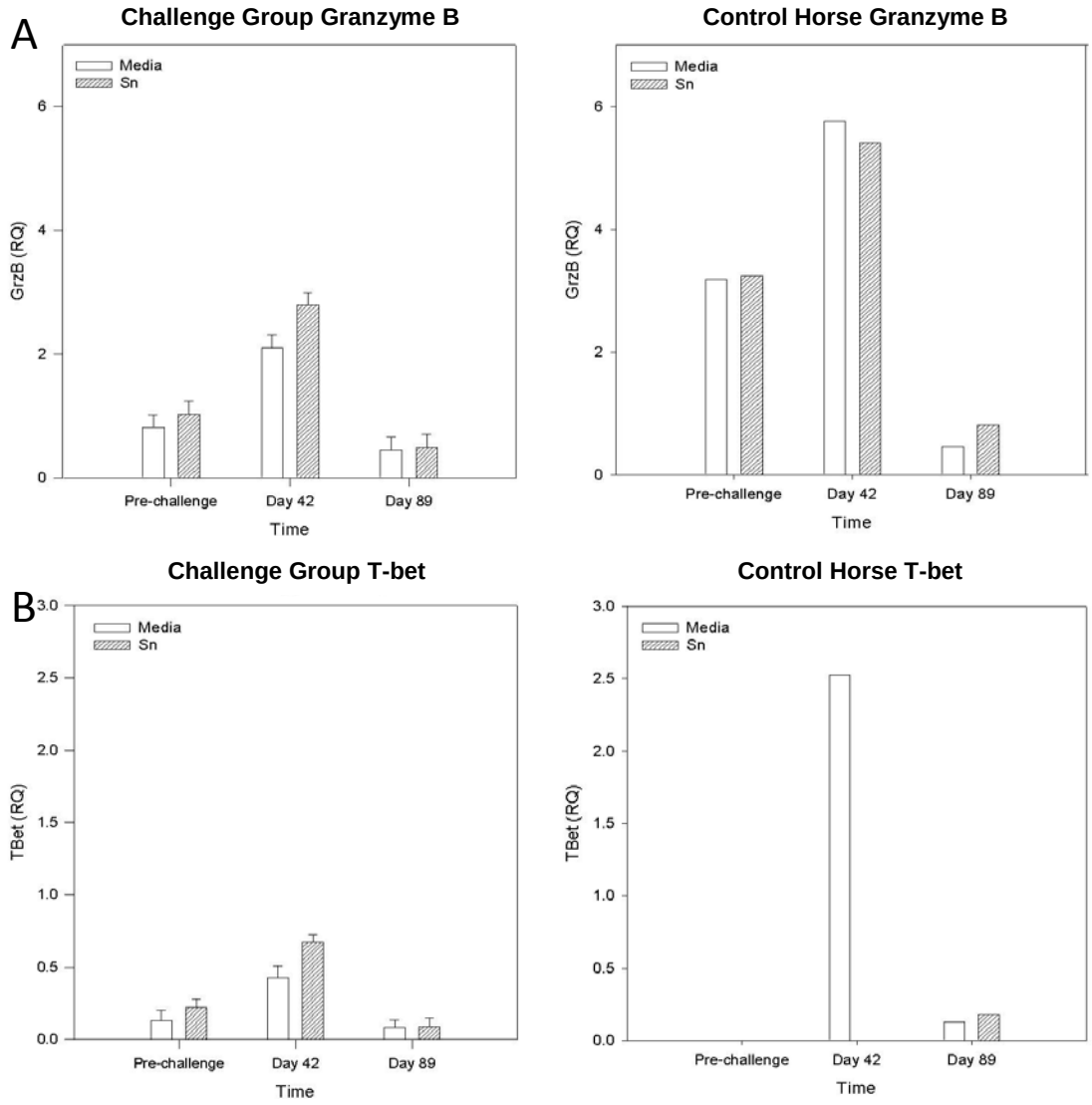

Figure 3.11. Relative quantification (RQ) of mRNA expression by Real-time PCR after *Sarcocystis neurona* stimulation. Heparinized blood was stimulated with SN3 strain *S. neurona* merozoites pre-challenge and on days 42 and 89 post inoculation. Mean (±SE) fold changes in expression of (A) Granzyme B and (B) T-bet for stimulated (Sn - striped bars) and non-stimulated (Media - white bars) blood of the challenge group and control horse. Raw RQ values are presented in graphs.

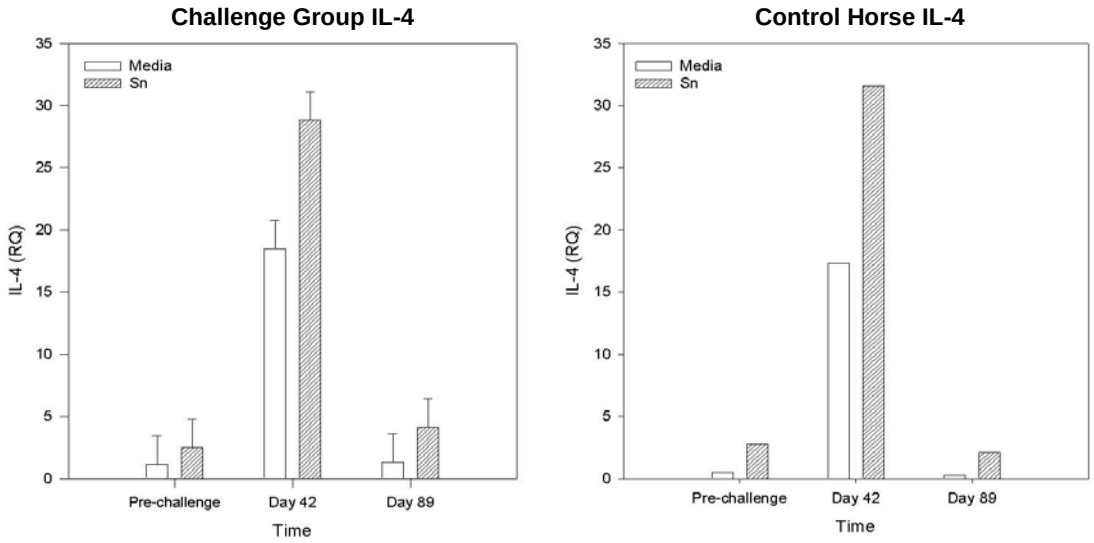

Figure 3.12. Relative quantification (RQ) of anti-inflammatory cytokine mRNA expression by Real-time PCR after *Sarcocystis neurona* stimulation. Heparinized blood was stimulated with SN3 strain *S. neurona* merozoites pre-challenge and on days 42 and 89 post inoculation. Mean (±SE) fold changes in expression of cytokine IL-4 for stimulated (Sn - striped bars) and non-stimulated (Media - white bars) blood of the challenge group and control horse. Raw RQ values are presented in graphs.

3.3.5. Merozoite Invasion Assay

The merozoite invasion assay was conducted to assess the viability of *S. neurona* merozoites after transportation at 37°C in a styrofoam container for two hours before being inoculated into the blood tubes. Time point 0 was used as the control and set at 100% invasion. After sitting at 37°C in the styrofoam container, the invasion dropped approximately 20% every two hours. Even after six hours, an appreciable proportion of the merozoites were still viable and able to infect the host cell monolayer.

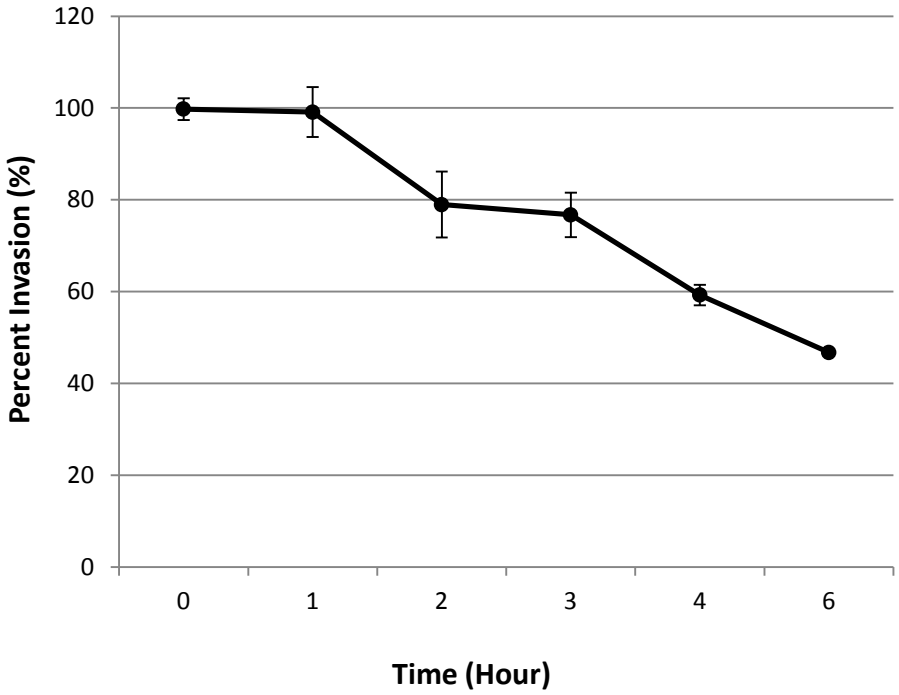

Figure 3.13. Percent invasion of *Sarcocystis neurona* F9F merozoites. After sitting in a 37°C styrofoam container, at different time points 2,000 *S. neurona* F9F merozoites were added to 3 wells of a 24-well plate containing a monolayer of BT cells. The plate was incubated at 37°C, and parasites were counted on day 4. Time point 0 was set at 100%. Values represent the average (±SE) of three wells for each time point.

3.4. Discussion

The infection method used in this study was modified from a previous EPM challenge model (Ellison and Witonsky, 2009; Ellison et al., 2004), which eliminates the natural migration of *S. neurona* through the gastrointestinal system by placing *S. neurona*-infected leukocytes directly into the bloodstream of the horse. Challenged horses developed mild to moderate neurological signs, and were seropositive for *S. neurona* on day 42. By day 89, the horses' serum antibody titers dropped, and the CSF lacked *S. neurona* antibodies. There was no histopathological evidence of a *S. neurona* infection. These results suggest that the challenged horses had an active infection on day 42, but had likely cleared the infection by the conclusion of the study.

Since the challenged horses did not appear to be infected by day 89, it is unclear why the horses continued to show increasing neurologic signs. Neurologic exams were taped and viewed by additional veterinarians, who agreed that the horses became more neurologic as the study progressed. Histological examination ruled out the possibility that the neurologic signs were caused by other factors (i.e., virus or bacterial infections, neurological disorders). Focal gliosis was observed in one section of the control horse's lumbar tissue, but this is non-specific for EPM. The minimal non-specific changes seen in the CNS of these horses have been observed in horses of other infection challenge models displaying mild to moderate neurological signs (Cutler et al., 2001; Saville et al., 2004b; Saville et al., 2001; Sofaly et al., 2002). One challenged horse displayed leukocytosis within the blood vessels of the lumbar spinal cord. Complete blood counts were not run on any of the horses. Therefore, it is unclear whether the horse had an

underlying systemic infection or if the leukocytosis was localized to that specific part of the spinal cord.

As with all other previous challenge models (Cutler et al., 2001; Ellison and Witonsky, 2009; Ellison et al., 2004; Fenger et al., 1997; Lindsay et al., 2000; Saville et al., 2004b; Saville et al., 2001; Sellon et al., 2004b; Sofaly et al., 2002), *S. neurona* was not identified histologically in the CNS tissue of the challenged horses. While a majority of the horses' spinal cords were removed for analysis, not all regions were observed. Given the focal nature of *S. neurona* infection, and the need for only a few organisms to cause disease (Dubey et al., 2001b), it is possible that *S. neurona* was present in tissue that was not examined under light microscopy. However, this seems unlikely as 4 of 5 challenged horses were negative for *S. neurona* antibodies in their CSF.

The SN3 strain of *S. neurona* expresses SnSAG1 and not SnSAG5, while strain SN4 expresses SnSAG5 but not SnSAG1. All challenged horses displayed reactivity to SnSAG1, and all but one reacted to SnSAG5. Therefore, both *S. neurona* strains infected the horses. Diagnostically, SnSAG2, SnSAG3, and SnSAG4 are usually more dependable markers for infection than SnSAG1 and SnSAG5 (Crowdus et al., 2008; Hoane et al., 2005). Isotypes IgGa and IgGb are associated with the induction of a Th1 response, while IgG(T) is involved with a Th2 response (Cunha et al., 2006; Hooper-McGrevy et al., 2003). All three isotypes being produced in the challenged horses coincided with the evident humoral and CMI responses elicited by the horses.

The CMI response is important in protecting the host against intracellular pathogens (Gazzinelli et al., 1994; Khan et al., 1997; Suzuki, 2002). Typically, a single cell type

(Th1 or Th2) will dominate the CMI response. Th1 immune responses are involved with intracellular pathogens (i.e., viruses, bacteria). The Th1 cells produce the cytokines IFN-γ, and IL-2. Th2 immune responses are linked to extracellular pathogens (i.e., multicellular parasites). The cytokine IL-4 is predominately produced by Th2 cells (Kidd, 2003).

IFN-γ has been shown to play an important role in the control of intracellular parasites, including *T. gondii*, *N. caninum*, *Leishmania* spp., and *Trypanosoma cruzi* (Khan et al., 1997; Lykens et al., 2010; Suzuki et al., 1988; Zambrano-Villa et al., 2002).When infected with *S. neurona*, IFN-γ KO mice developed terminal encephalitis, while infection of immunocompetent mice did not result in infection (Dubey and Lindsay, 1998; Witonsky et al., 2003a). In this study, challenged horses had an increase in IFN-γ expression on day 42, with a statistical difference of IL-2 expression between the sample days. Due to the production of these cytokines, and without an increase in IL-4 levels, it appears that the horses were producing a Th1 response. The initiation of a Th1 type response in the challenge horses may explain why they were able to clear the infection. Studies of naturally infected EPM horses have shown an increase of IL-4 expression with IFN-γ suppression, thus indicating a Th2 immune response (Spencer et al., 2005). As the Th2 type response is not favorable for the clearance of intracellular parasites (Zambrano-Villa et al., 2002), this could indicate the reason for infection. Further investigation is needed to understand the factors behind a Th1 or Th2 immune response to *S. neurona*.

The outcome of this study was different than that of the model from which it was modified (Ellison and Witonsky, 2009; Ellison et al., 2004). Possible explanations may lie in minor procedural differences. In this study, the merozoites were transported for 2

hours prior to inoculating the blood samples. However, the invasion assay indicated that at 2 hours there was only a 20% decrease in the capability of the merozoites to infect cells of the monolayer. Therefore, it is reasonable to believe that 800,000 *S. neurona* merozoites were still viable to invade cells when inoculated into the blood tubes. In this study, blood tubes were incubated for 4 hours as opposed to overnight by Ellison and Witonsky (2009). Additionally, parasites were only given 4 times at a high dosage in this study, as opposed to Ellison and Witonsky (2009) administering low amounts over more consecutive days (100-10,000 merozoites for 14-15 days).

The infection model used in this study did not cause horses to develop clinical EPM. There has yet to be an EPM model able to recreate the degree of disease seen in naturally infected horses. Further research is needed to better understand the pathogenesis of the disease and the interaction of *S. neurona* and the horse immune system.

CHAPTER FOUR

Conclusion

The studies described in this thesis investigated factors contributing to the development of EPM in the horse. In the first study, an attempt to look at potential susceptibility of horses to EPM was performed using a GWAS on FFPE tissue. The DNA extracted from the FFPE tissues was not adequate for SNP analysis, however, only yielding usable results in 5 of 24 horses. Tissue age appeared to be a factor in the viability of the DNA. Tissue that was older than 14 months showed a dramatic drop in call rate. It seems likely that use of FFPE tissues for GWAS will not be a practical method for an EPM study, as the UK VDL does not process enough cases annually to develop a reasonable sample size. Collaboration with other veterinary diagnostic laboratories would be needed to create an appropriate sample size of FFPE samples. Additionally, samples could be processed soon after case is received, and DNA stored until a sufficient sample size is collected.

Illumina has developed a restoration solution to be used on DNA damaged in FFPE tissue (Pokholok et al., 2010). Through the use of polymerases, degraded DNA is repaired, leaving strands of DNA that do not contain the damaged bases. Strands can then be ligated together and run on an Infinium® HD Assay (Illumina). To date this technology has only been optimized for use on human SNP chips. In the future, it might be possible to use the restoration solution on damaged DNA extracted from equine FFPE samples before running them on an equine SNP chip. This may result in increased call rates, making the archived FFPE samples usable in a GWAS on EPM susceptibility.

The second study tested the use of a horse EPM model that involves injecting whole blood infected with *S. neurona* merozoites directly into the bloodstream of horses. Neurological examinations along with humoral and CMI responses were evaluated. At the conclusion of the study, horses displayed mild to moderate neurological signs and appeared to have cleared the *S. neurona* infection. Challenged horses had an increased expression of IFN-γ and IL-2, indicating a Th1 immune response. The severe clinical signs seen with natural infections were not observed in these challenged horses. Therefore, the infection model used in this experiment was not effective in replicating clinical EPM.

Multiple attempts have been made to develop a working equine EPM model, but each has been unable to replicate the disease seen in naturally infected horses. There appears to be an underlying component needed to cause the disease that has not been identified with these challenge models. Questions regarding the interaction between *S. neurona* and the horse immune response should be addressed prior to attempting another EPM model. Additional studies are needed to confirm the relationship between IFN-γ and IL-4 expression in horses infected with *S. neurona*. Other factors that may be involved include the horses' genetics, stress level, and concurrent infections. As well, the *S. neurona* strain virulence and mechanisms of immunosuppression may play a role. Greater understanding of the pathogenesis of EPM could lead to improved diagnostics, treatment, identification of potential susceptible horses, and, ultimately, prevention of this devastating disease.

APPENDIX A

List of Abbreviations

AMV	Avian myelobastosis virus
ANOVA	Analysis of variance
AVMA	American Veterinary Medical Association
β-GUS	Beta-glucuronidase
BT	Bovine turbinate
C	Cervical
cDNA	Complementary DNA
CMI	Cell-mediated immunity
CNS	Central nervous system
CSF	Cerebrospinal fluid
CVM	Cervical vertebral malformation
DAB	Diaminobenzidine tetrahydrochloride
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dpi	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPM	Equine protozoal myeloencephalitis
ETCR	East Tennessee Clinical Research, Inc.
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FFPE	Formalin-fixed, paraffin-embedded
GrzB	Granzyme B
GWAS	Genome-wide association studies
H&E	Hematoxylin and eosin
HRP	Horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IH	Intermediate host
IHC	Immunohistochemical
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
KO	Knockout
L	Lumbar
mAb	Monoclonal antibody
MCP1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MSMD	Mendelian susceptibility to mycobacterial disease
NK	Natural killer
NSAID	Nonsteroidal anti-inflammatory drug
OD	Optical density
PBS	Phosphate buffered saline
PBST	PBS with 0.05% Tween 20

PCR	Polymerase chain reaction
PI	Post inoculation
PID	Primary immunodeficiencies
PMA/I	Phorbol myristate acetate/ionomycin
RNA	Ribonucleic acid
RQ	Relative quantification
RT	Room temperature
S	Sacral
SCID	Severe combined immunodeficiency
SE	Standard error
SNP	Single nucleotide polymorphism
SnSAG	*Sarcocystis neurona* surface antigen
T	Thoracic
UK VDL	University of Kentucky Veterinary Diagnostic Laboratory
US	United States
USDA	United States Department of Agriculture
YFP	Yellow fluorescent protein

APPENDIX B

Neurologic Examination Results

Table B.1. Horse 463 – frequency distribution of neurologic scores.

STUDY DAY	SCORE 0	SCORE 1	SCORE 2	SCORE 3	ADDITIONAL OBSERVATIONS
-2	60	4	0	0	N/A
7	60	4	0	0	Cranial Nerves not examined
12	46	9	9	0	No obvious cranial nerve deficits
21	60	4	0	0	Gait worsened with head elevated (at a walk)
28	57	7	0	0	Gait worsened with head elevated
35	54	10	0	0	Gait worsened with head elevated
42	28	28	8	0	Hyporeflexive menace response; right ventrolateral strabismus; right ear droop; right head tilt. Gait worsened with head elevated and walking on a slope
49	54	8	2	0	Gait worsened with head elevated
63	24	29	10	1	Gait worsened with head elevated
77	22	24	15	3	Gait worsened with head elevated and walking on a slope
89	0	32	30	2	Gait worsened with head elevated and walking on a slope

Table B.2. Horse 464 – frequency distribution of neurologic scores.

Study Day	Score 0	Score 1	Score 2	Score 3	Additional Observations
-2	59	5	0	0	N/A
7	62	2	0	0	Cranial Nerves not examined
12	31	24	9	0	Gait worsened with head elevated and walking on a slope
21	57	7	0	0	Gait worsened with head elevated
28	52	12	0	0	Gait worsened with head elevated
35	54	10	0	0	N/A
42	4	42	18	0	Gait worsened with head elevated and walking on a slope; no change when blindfolded
49	44	11	9	0	Gait worsened with head elevated
63	36	22	6	0	Gait worsened with head elevated
77	28	28	8	0	Gait worsened with head elevated
89	16	24	24	0	Gait worsened with head elevated and walking on a slope

Table B.3. Horse 465 (Control) – frequency distribution of neurologic scores.

STUDY DAY	SCORE 0	SCORE 1	SCORE 2	SCORE 3	ADDITIONAL OBSERVATIONS
-2	64	0	0	0	N/A
7	55	8	1	0	Cranial Nerves not examined; Gait worsened with head elevated
12	33	17	14	0	(Horse resisted head elevation)
21	54	8	2	0	Gait worsened with head elevated
28	47	17	0	0	Gait worsened with head elevated
35	45	14	5	0	Gait worsened with head elevated
42	44	15	5	0	No change when blindfolded
49	30	23	11	0	Gait worsened with head elevated
63	38	18	8	0	Gait worsened with head elevated
77	24	26	14	0	Gait worsened with head elevated
89	40	16	8	0	Gait worsened with head elevated

Table B.4. Horse 466 – frequency distribution of neurologic scores.

STUDY DAY	SCORE 0	SCORE 1	SCORE 2	SCORE 3	ADDITIONAL OBSERVATIONS
-2	63	1	0	0	Moves very well
7	53	10	1	0	Cranial Nerves not examined
12	28	14	21	1	Gait worsened with head elevated
21	50	14	0	0	Gait worsened with head elevated
28	50	9	5	0	Gait worsened with head elevated and walking on a slope
35	39	22	3		Gait worsened with head elevated
42	44	17	3	0	No change when blindfolded
49	28	22	14	0	Gait worsened with head elevated and walking on a slope
63	35	17	12	0	Gait worsened with head elevated
77	44	15	5	0	Gait worsened with head elevated
89	30	6	24	4	Gait worsened with head elevated and walking on a slope

Table B.5. Horse 467 – frequency distribution of neurologic scores.

STUDY DAY	SCORE 0	SCORE 1	SCORE 2	SCORE 3	ADDITIONAL OBSERVATIONS
-2	52	12	0	0	Mild ataxia in circles- trots ok but weak at stopping
7	58	5	1	0	Cranial Nerves not examined
12	36	28	0	0	Gait worsened with head elevated (pacing). Muzzle deviated while eating.
21	51	13	0	0	Gait worsened with head elevated
28	42	17	5	0	Gait worsened with head elevated and walking on a slope
35	45	16	3	0	Gait worsened with head elevated
42	18	36	10	0	Gait worsened with head elevated and walking on a slope; no change when blindfolded
49	30	23	11	0	Gait worsened with head elevated and walking on a slope
63	22	26	14	2	Gait worsened with head elevated
77	38	14	11	1	Gait worsened with head elevated
89	0	4	50	10	Gait worsened with head elevated and walking on a slope

Table B.6. Horse 468 – frequency distribution of neurologic scores.

STUDY DAY	SCORE 0	SCORE 1	SCORE 2	SCORE 3	ADDITIONAL OBSERVATIONS
-2	59	5	0	0	Backs well, trots well; trots very nice
7	7	6	1	0	Cranial Nerves not examined; Gait worsened with head elevated
12	32	28	4	0	N/A
21	56	8	0	0	Gait worsened with head elevated
28	42	18	4		Gait worsened with head elevated and walking on a slope
35	40	14	10	0	Gait worsened with head elevated and walking on a slope
42	0	28	30	6	Gait worsened with head elevated and walking on a slope; no change when blindfolded
49	20	25	19	0	Gait worsened with head elevated and walking on a slope
63	14	34	12	4	Gait worsened with head elevated
77	32	22	10	0	Gait worsened with head elevated
89	4	18	31	11	Gait worsened with head elevated and walking on a slope

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