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CONTROLLED INTERVENTIONS USING PYRANTEL TARTRATE TO PREVENT *SARCOCYSTIS NEURONA* INFECTION IN HORSES

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CONTROLLED INTERVENTIONS USING PYRANTEL TARTRATE TO PREVENT *SARCOCYSTIS NEURONA* INFECTION IN HORSES

By

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ABSTRACT

CONTROLLED INTERVENTIONS USING PYRANTEL TARTRATE TO PREVENT *SARCOCYSTIS NEURONA* INFECTION IN HORSES

By

Mary Gordon Rossano

This dissertation describes two studies investigating the efficacy of pyrantel tartrate in preventing infection with the protozoan parasite *Sarcocystis neurona* in horses. *Sarcocystis neurona* is the primary etiologic agent of equine protozoal myeloencephalitis (EPM), a neurological disease of horses and ponies of the Americas. A third study described here details the culture of *S. neurona* from the blood of an immunocompetent horse.

In the first study, 24 horses were challenged with sporocysts of *S. neurona*. Twelve of the horses received daily doses of pyrantel tartrate, 12 received a look-alike placebo pellet. Production of serum antibodies against *S. neurona* was the main outcome of interest, as this is evidence the parasite was not killed in the gastrointestinal tract by the drug and that the parasite had penetrated host defenses. Production of antibodies in

cerebrospinal fluid (CSF) was a second outcome of interest.. At the end of the study there was no significant difference between treatment groups with regard to the proportion of horses testing positive or the days to seroconversion.

In the second study, a field intervention trial was conducted on three Michigan horse farms to determine whether daily administration of pyrantel tartrate could prevent infection with *S. neurona* in horses when the drug was used in an on-farm setting, according to label instructions for Strongid C® 2x. The outcome of interest was the production of serum antibodies to *S. neurona*. Horses were screened negative by immunoblot for serum antibodies to *S. neurona* and allocated into two treatment groups: pyrantel tartrate and placebo. Ten horses were monitored for seroconversion for 6 months. The results of the study were inconclusive, due to the small sample size, and no treatment effect was detected.

In the third study, six yearling colts given daily doses of *S. neurona* sporocysts, were selected for attempted culture of *S. neurona* from whole blood. Two 10 ml tubes of blood in EDTA were collected from each horse. Plasma was removed from the tubes and processed to remove fibrinogen and cultured on equine dermal cells. Thirty-eight days later the culture from one horse was positive. To our knowledge, this is the first report of parasitemia in an immunocompetent infected with *S. neurona* sporocysts.

This dissertation is dedicated to my husband, Greg Wood, for his encouragement and patience over the 3 ½ years that have passed since I began this degree, especially during the 6 months we spent in Washington. It is also dedicated to our parents, Helen H. Rossano and John and Ruth Wood, for their support and understanding. Finally, I dedicate this dissertation to the future of our son or daughter, not yet born, and all the promise that future holds.

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OVERVIEW

Chapters 2, 3 and 4 in this thesis are written in a format suitable for publication independently. Thus, those chapters each have an abstract, introduction, methods and discussion. Chapter 1, which is a literature review, does not follow this format. A list of literature cited concludes each chapter.

Chapter 1 is a literature review of equine protozoal myeloencephalitis (EPM). It includes the epidemiology, clinical signs, diagnosis and treatment of the disease, as well as a description of the causative organism, *Sarcocystis neurona*. Chapter 1 also contains the rationale, objectives and hypotheses for the studies described here. Chapter 2 describes an experimental challenge study in which horses were dosed with *S. neurona* in order to test the efficacy of pyrantel tartrate in preventing infection with *S. neurona*. Chapter 3 is a field intervention trial in which pyrantel tartrate was tested to determine whether it can prevent natural infection with *S. neurona* in horses in an on-farm setting. Chapter 4 describes the culture of *S. neurona* from the blood of an immunocompetent horse. The dissertation concludes with Chapter 5, which is an overall summary and synthesis of the findings of this research. Appendices follow Chapter 5.

CHAPTER 1

INTRODUCTION

RATIONALE AND JUSTIFICATION FOR THE STUDY:

Equine protozoal myeloencephalitis (EPM), a multi-focal neurological disease caused by the protozoan parasite *Sarcocystis neurona*, has imposed a significant health threat to horses, and economic and emotional costs and their owners (MacKay, 1997). The economic impact of EPM on the horse industry has been estimated between $50 and $100 million annually in the United States (Dubey et al., 2001). The majority of this cost has been incurred by horse owners pursuing diagnosis and treatment of EPM. Unfortunately, even when horses respond favorably to treatment, the chance for return to their prior level of performance appears to be, at best, 50% (MacKay 1997; Dubey 2001). When faced with a disease with such an uncertain outcome, preventive measures are of great interest and have a greater potential economic impact, than improved treatments that are administered once clinical signs have developed.

In areas of the western hemisphere where the definitive host (the Virginia opossum, *Didelphis virginianus*) harboring *S. neurona* is found, detection of serum antibodies to *S. neurona* indicates that 30-60% of horses are, or have been, infected with the parasite (Bentz et al 1997; Blythe et al 1997; Saville et al 1997; Tillotson et al 1999; Rossano et al 2001). Consequently, horse owners are interested in any preventive measures that could possibly decrease the risk of their horses developing EPM.

Despite a concerted research effort at several universities leading to improved diagnostic tests and treatments for EPM, there has been little progress in the understanding of its prevention (Dubey 2001; Rossano et al 2001). In fact, equine veterinarians have little advice to offer concerned clients besides restricting wildlife access to feeds and trapping and removing opossums from their farms. Thus, it is not surprising that some concerned horse owners are using the recently released *S. neurona* vaccine (Fort Dodge Animal Health, conditional licensure awarded December, 2000), despite the fact that the efficacy of the vaccine has not been demonstrated. While the vaccine offers some hope in the prevention of EPM, identification of other effective preventive measures against *S. neurona* infection would be of great interest and benefit to the equine industry.

In 1999, our research group was asked to submit a research proposal to Pfizer, Inc. to investigate the validity of anecdotal reports they had received from equine practitioners that EPM was seen less often in horses that were maintained on their daily deworming product, Strongid C®. The active ingredient in Strongid C® is pyrantel tartrate. If pyrantel tartrate proved useful in preventing healthy horses from becoming infected with *S. neurona* it would be a great benefit to horses and their owners in the western hemisphere. Thus, we undertook several studies to determine the efficacy of daily administration of pyrantel tartrate in preventing infection of horses by *S. neurona*.

PROBLEM STATEMENT:

Sarcocystis neurona is a ubiquitous parasite in areas of the western hemisphere where opossums reside, and many horses in those regions are infected with the parasite, a

few of which go on to develop EPM (Dubey et al., 2001a). Preventive options are limited in the face of high exposure to the parasite, and there is great interest among horse owners for EPM prevention. Anecdotal reports from field veterinarians suggest that daily administration of pyrantel tartrate may prevent infection of horses with *S. neurona*. Therefore, research to investigate the purported ability of daily pyrantel tartrate to prevent EPM could address an important concern of the horse industry.

STUDY HYPOTHESIS AND OBJECTIVES:

The **hypotheses** tested were:

> H_1: Daily pyrantel tartrate administration prevents infection with *S. neurona* in horses.
>
> H_2: Daily pyrantel tartrate administration delays infection with *S. neurona* in horses.
>
> H_3: Daily pyrantel tartrate administration prevents infection with *S. neurona* in horses under field conditions.

And, as an additional investigation of how *S. neurona* infects horses,

> H_4: *S. neurona* can be detected in the blood of immunocompetent horses.

Three **objectives** were conducted to test the stated hypotheses.

1. To determine whether daily administration of pyrantel tartrate decreases the risk of seroconversion during a period of repeated challenge of seronegative horses with *S. neurona* sporocysts.
2. To conduct a controlled field intervention study on equine breeding farms to determine whether daily administration of pyrantel tartrate decreases the risk of seroconversion in young horses during the first year of life.
3. To culture live *S. neurona* from the blood of an immunocompetent horse.

LITERATURE REVIEW

1. EQUINE PROTOZOAL MYELOENCEPHALITIS:

Equine protozoal myeloencephalitis (EPM) is a progressive, multi-focal neurological disease of the horse. It was originally described in the 1960's as focal myelitis-encephalitis because affected areas of the central nervous system included both the spinal cord and the brain (Rooney et al., 1969). The disease was renamed in the 1970's when protozoan parasites were detected in spinal cord lesions of affected horses (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974).

2. CLINICAL SIGNS:

The clinical signs of EPM are highly variable; it can resemble other neurological diseases, due to the multi-focal locations of the lesions (MacKay 1997; Dubey et al., 2001; Furr et al., 2002). Signs that may be apparent include: mild lameness, gait deficits, head tilt, facial paralysis, limb ataxia, spasticity, weakness, stumbling or falling, recumbency, atrophy of focal muscle groups (muscles that are enervated by damaged nerves), abnormal sweating, urinary incontinence and constipation or colic (MacKay 1997; Dubey et al., 2001; Furr et al., 2002). Usually the lameness or paralysis is asymmetrical; asymmetry of clinical signs is helpful in differentiating EPM from other neurological diseases (MacKay 1997; Dubey et al., 2001; Furr et al., 2002).

The rate at which EPM progresses differs by individual case. It can appear as a subtle lameness or gait abnormality in its early stages (Mayhew and Greiner, 1986; MacKay et al., 1992). Later, the horse may become ataxic and weak (Rooney et al.,

1969; Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987; MacKay et al., 1992). Ultimately, the horse can become recumbent or be found sitting in a dog-like position (Rooney et al., 1969; Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987; MacKay et al., 1992). In some cases the disease is slow and chronic, and the lesions may remain quiescent for months or years without treatment (Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987; MacKay et al., 1992). In other instances the rate of progression is very rapid, with the lesions fulminating in hours or days (Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987).

Other diseases that cause peripheral or central nerve damage can present signs that are similar to those for EPM. In making a diagnosis of EPM, a clinician must be differentiate EPM from: West Nile virus encephalitis, compressive myelopathy (wobblers), equine herpes myelitis, multi-focal bacterial infections, rabies, tetanus, poly neuritis equi, neoplasia, eastern and western equine encephalitis, equine degenerative myeloencephalopathy, leukoencephalomalacia (moldy corn poisoning), helminth parasite migration and head or spinal trauma (Rooney et al., 1969; Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987; MacKay et al., 1992; Furr et al., 2002.). Of course, it is possible that a horse may have more than one neurological disease at once (e.g. EPM and wobblers); thus, identification of one problem may not necessarily rule out all others.

3. CAUSATIVE AGENT:

In early investigations, after protozoa were identified in neural tissue of affected horses, *Toxoplasma gondii* was considered to be a possible causative organism of the disease (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974). In Brazil, *T. gondii* infection has been associated with equine neurological disease, but it was likely that it was mistakenly associated with cases of EPM (Mayhew et al., 1978; Mayhew and Greiner, 1986; Simpson and Mayhew, 1980; Masri et al, 1992). By 1980, there was strong evidence that the causative agent of EPM was a *Sarcocystis spp*, not *Toxoplasma gondii* (Simpson and Mayhew, 1980). In 1991, the parasite was cultured from central nervous system tissue from a naturally infected horse, described and named *Sarcocystis neurona* (Dubey et al., 1991). *Sarcocystis neurona* was later confirmed as the primary etiologic agent of EPM (Bowman et al., 1992; Fenger et al., 1994; Dubey and Lindsay 1998; Dubey et al., 1998). Despite the predominance of *S. neurona*-related EPM cases, four cases of EPM have been attributed to *Neospora spp.* infection (Marsh et al., 1996; Daft et al., 1997; Hamir et al., 1998; Cheadle et al., 1999). In one of the cases, the parasite was cultured from neural tissue and was determined to be distinct from *Neospora caninum* (Marsh et al., 1998b). This new species of *Neospora* was described, characterized, and named *N. hughesi* (Marsh et al., 1998). Although this was a noteworthy discovery, additional cases of *Neospora*-induced myeloencephalitis must be reported before *Neospora spp.* can be considered a significant cause of EPM.

Sarcocystis species have an obligatory, heteroxenous life cycle (Levine 1985; Urquhart et al., 1996). That is, in order to reproduce, the parasite must go through replication and development within two different hosts: an intermediate host and a

definitive host. In the intermediate host, the terminal asexual stage is formed, and in the definitive host, sexual reproduction occurs (Levine 1985; Urquhart et al., 1996). The life cycle of *Sarcocystis* species is circular, and this description will start with the definitive host.

Cysts (sarcocysts) in muscle tissue of the intermediate host are consumed by the definitive host, usually a carnivore (Levine 1985). The cyst wall is digested, releasing bradyzoites that actively penetrate the host's intestinal epithelium, where they undergo gametogeny and develop into the sexual stages, microgametes and macrogametes (Levine 1985). The macrogamete is fertilized by the microgamete, and the resulting zygote matures to become an oocyst that contains two sporocysts (Levine 1985). The oocyst is released from its host epithelial cell, in most cases the oocyst wall is ruptured as it travels down the intestine, freeing the sporocysts which are then excreted in the feces (Urquhart et al., 1996). At this stage, each sporocyst contains four sporulated sporozoites capable of infecting an appropriate intermediate host or an aberrant host (Levine 1985).

The intermediate host, usually a prey species, becomes infected when it ingests fecal material, or food or water contaminated with feces, which contain sporocysts from the definitive host (Levine 1985). Upon ingestion, the sporocysts release their sporozoites in the intermediate host's gastrointestinal tract, which then migrate to endothelial cells of the host's internal organs (Urquhart et al., 1996). The tissues affected depend on the particular *Sarcocystis* species and intermediate host infected (Levine 1985; Urquhart et al., 1996). In most cases, the parasite undergoes two rounds of asexual schizogony and forms tachyzoites, and eventually, motile merozoites (Urquhart et al., 1996). Merozoites are subsequently released from schizonts, then travel in circulating lymphocytes to

muscle tissue (Urquhart et al., 1996). There they undergo fixed rounds of merogony (asexual reproduction) and develop into the terminal asexual stage, bradyzoites, in intramuscular cysts (Urquhart et al., 1996). The sarcocysts are tightly packed with bradyzoites, which are infective to the appropriate definitive host (Levine 1985). The cycle begins anew when the intermediate host is eaten as prey or carrion, and the bradyzoites emerge in the carnivorous host's intestine. (Levine 1985; Urquhart et al., 1996).

Sarcocystis species are usually very specific to their hosts (Levine 1985; Urquhart et al., 1996). Although aberrant infections are possible, usually *Sarcocystis* spp. cannot form terminal sexual or asexual stages in an aberrant host (Levine 1985). One notable exception to this tendency is *Sarcocystis neurona*, which is thus far found to be specific to its definitive host, the opossum (*Didelphis virginiana*)(Dubey, et al., 2001a), but has been shown to utilize a variety of unrelated species (the nine-banded armadillo (*Dasypus novemcinctus*) (Cheadle et al., 2001a), the domestic cat (*Felis domesticus*) (Dubey et al.,2000), the striped skunk (*Mephitis mephitis*) (Cheadle et al., 2001b), the raccoon (*Procyon lotor*) (Dubey et al., 2001c) and the sea otter (*Enhydra lutris*) (Dubey et al., 2001b) as its intermediate hosts. Another exception to the tendency of *Sarcocystis* spp. specificity to their intermediate host is *Sarcocystis falcatula*, which is specific to its definitive host, also the opossum (*Didelphis virginiana*), but has been shown to utilize a variety of bird species as its intermediate host (Box and Smith, 1982).

Control of *Sarcocystis* spp. can be problematic. Tissue cysts are enclosed in a thick wall comprised of 2 membranes (Levine, 1985). Bradyzoites within the cysts can survive incomplete cooking, but are killed by freezing (Levine, 1985). *Sarcocystis* spp.

sporocysts are incased in a relatively impervious wall and are extremely well adapted to survive in the environment (Levine, 1985). *Sarcocystis gigantea* sporocysts have been shown to have remarkable resistance to freezing, desiccation, and various chemical agents, including 2.5% hydrochloric acid, 10% formalin, and 0.5% ammonia solution (McKenna and Charleston, 1992). *Sarcocystis neurona* sporocysts can survive a temperature of 50°C for 1 hour (Dubey et al., 2002). Treatment with bleach (10, 20, and 100%), 2% chlorhexidine, 1% betadine, 5% o-benzyl-p-chlorophenol, 12.56% phenol, 6% benzyl ammonium chloride, and 10% formalin does not kill them (Dubey et al., 2002). Exposure to undiluted ammonium hydroxide (29.5% ammonia) for 1 hour kills sporocysts, as does heating them to 55°C for 15 min and 60°C or more for 1 minute (Dubey et al., 2002). Unfortunately, none of the lethal treatments is practical for disinfecting barns or feedstuffs.

The horse is a natural intermediate host to three *Sarcocystis* species. *S. bertrami, S. equicanis* and *S. fayeri* form cysts in the muscle tissue of equids and complete their life cycles in canid definitive hosts (Levine, 1986). The distribution of *S. bertrami* and *S. equicanis* is speculated to be worldwide; *S. fayeri* has only been reported in North America (Levine, 1985). It is speculated that the three species may be synonomous (Levine, 1985). These species of *Sarcocystis* are not normally pathogenic to either their definitive or intermediate hosts, and in Britain, where the practice of feeding hunt horses to hounds exists, the infection rate in horses has been estimated to be between 60 and 70 percent (Edwards, 1984).

Solving the life cycle of *S.* neurona was a slow process that involved researchers at a number of different centers. *S. neurona* was first shown to utilize the opossum as its

definitive host in 1995 (Fenger et al., 1995) but it was not until the early 21st century that the intermediate hosts were identified. In 1995, a phylogenetic study suggested that *S. neurona* was synonymous with *S. falcatula* (Dame et al., 1995), which had been reported to use the opossum as its definitive host, and various species of birds as intermediate hosts (Box and Smith, 1982). Later investigations revealed the two species of *Sarcocystis* did not infect the same intermediate host species. *S. neurona* was shown to not be infective to budgerigars (*Melopsittacus undulates*), while *S. falcatula* proved to be highly pathogenic to buderigars (Marsh et al., 1997b). *S. neurona* produced neurological disease in immunocompromised nude and gamma-interferon knockout mice (Marsh et al., 1997a; Dubey et al., 1998a; Dubey et al., 1998b), but *S. falcatula* did not (Dubey et al., 1998a; Dubey et al., 1998b). In an infection challenge of *S. falcatula* in horses, no clinical disease was produced (Cutler et al., 1999). A series of investigations revealed that the opossum serves as the definitive host for at least 3 distinct species of *Sarcocystis*: *S. falcatula, S. neurona,* and *S. speeri* (Dubey et al., 1998a; Dubey et al., 1998b; Dubey and Lindsay, 1999).

Horses are aberrant hosts to *S. neurona,* in which the parasite cannot undergo its final stage of schizogony (Dubey et al., 2001a). The schizonts formed during the course of EPM contain merozoites (Dubey et al., 2001a). The terminal asexual form, the sarcocyst, has not been found in infected horses (Dubey et al., 2001a). A carnivore cannot become infected by eating *S. neurona* merozoites, thus the horse is a dead-end host (MacKay et al., 1992). Recently, however, there was a report of a young colt with neurological disease in which both mature schizonts in the brain and spinal cord and mature sarcocysts in the tongue were found (Mullany et al., 2003). Electron microscopy

revealed that the sarcocysts had characteristics that were consistent with published characteristics of *S. neurona*. Testing for genetic markers showed that the sarcocysts produced *Sarcocystis*-specific PCR products and these products exhibited banding patterns characteristic of *S. neurona*. Further studies will be required to determine whether horses are true intermediate hosts to *S. neurona* or if this colt was abnormally susceptible due to his young age, or some form of immunosuppression.

Since 1997, a number of experimental infection studies have been performed in which specific pathogen free horses were given doses of *S. neurona* sporocysts in an attempt to reproduce clinical EPM. (Fenger et al. 1997, Saville et al. 2001, Cutler et al. 2001, Sofaly et al., 2003). In the first study (Fenger et al., 1997), the investigators were operating under the assumption that *S. neurona* was synonymous with *S. falcatula* and thus produced sporocysts by feeding tissue from birds infected with sporocysts harvested from feral opossums to specific pathogen free opossums. These sporocysts, most likely *S. falcatula*, were pooled with sporocysts from feral opossums and administered to foals in doses ranging from 1×10^6 to 2×10^7, with some foals receiving repeat doses, and one foal receiving daily doses of dexamethasone for 2 weeks to induce immunosuppression. Despite the fact that sporocysts were counted, it was not possible to ascertain the dose of *S. neurona* given to the foals, due to the likely presence of *S. falcatula* sporocysts in the inoculum. Foals first produced serum antibodies to *S. neurona* from 19-42 days and CSF antibodies 28 days after the first inoculation, with 1 seropositive foal failing to produce CSF antibodies. Neurological signs were reported in four of five foals and at post mortem examination microscopic inflammatory lesions suggestive of EPM were found in neural tissue from 3 of the foals with neurological signs, but not the fourth. That foal had

spinal cord lesions that were more suggestive of spinal cord compression. Two uninfected control foals remained seronegative and neurologically normal throughout the study. No *S. neurona* was identified in or cultured from any neural tissue specimen tested in this study (Fenger et al., 1997).

Another published equine infection study studied the progression of immunoconversion in horses with and without dexamethasone-induced immunosuppression (Cutler et al., 2001). When this study was conducted, molecular markers identified by Tanhauser et al. (1999) could be used to identify pure infections of *S. neurona* in feral opossums, enabling investigators to administer known doses of sporocysts to experimentally infected horses. Yearling specific pathogen free horses were divided into two treatment groups, in which four horses received daily dexamethasone and 4 horses did not. Both groups were challenged with with 7 daily consecutive doses of 5×10^5 sporocysts; two additional horses were maintained as unchallenged controls. The horses were isolated in stalls for 11 days following dosing, then transferred to a group enclosure. Horses were observed for neurological signs and serum and CSF were tested for antibodies to *S. neurona*. In addition, attempts were made to identify or isolate *S. neurona* from whole blood, neural tissue, heart, lung, liver, kidney spleen, thyroid, tongue, esophagus, stomach, small and large intestine, mesenteric lymph nodes, and skeletal muscle. Horses produced serum antibodies 17-32 days and CSF antibodies 17-61 days after the first inoculation. Horses receiving dexamethasone tended to immunoconvert sooner than those that did not, but this difference was not statistically significant. One control horse seroconverted 75 days after the experimental horses were challenged. Mild neurological signs were observed in horses in both treatment groups,

but the mean scores for each treatment group never exceeded 1.5 (on a scale of 0-4) for either group, and the horses not receiving dexamethasone appeared to be improving without treatment as time progressed. Microscopic inflammatory lesions were found in some challenged horses, but were not considered characteristic of active EPM. No attempt to culture or detect *S. neurona* post mortem was successful. The authors suggested that despite the fact that the horses were challenged with extremely high doses of sporocysts, they experienced transient CNS infections that were progressing toward clinical recovery without antiprotozoal treatment. (Cutler et al., 2001).

Two studies conducted in Ohio attempted to induce EPM in horses experimentally infected with *S. neurona* after being subjected to the stress of long-distance transport (Saville et al., 2001; Sofaly et al., 2003). In the first study (Saville et al. 2001), sporocysts used in the experimental challenges were obtained from 24 wild-caught opossums and pooled into one inoculum. Because the opossums likely had mixed *Sarcocystis* infections, the *S. neurona* content was estimated using a bioassay with gamma-interferon knockout mice. The lethal mouse dose was determined from the proportion of mice in each group that developed neurological disease, and it was estimated that the horses were administered at least 80,000 times the lethal mouse dose. Twelve horses were divided into four groups of three, with one group receiving sporocysts immediately following a 55-hour trailer ride, one group receiving sporocysts after having 14 days to acclimate following a 55-hour trailer ride, one group receiving sporocysts and daily dexamethasone for three days after having 14 days to acclimate following a 55-hour trailer ride and a control group received no sporocysts after a 55-hour trailer ride. Horses were evaluated for neurological signs by blinded equine

clinicians who were not aware of dose treatment assignments. Horses in the transport-stressed group seroconverted 2 weeks post-inoculation, horses in the group that had 14 days to acclimate seroconverted 2-4 weeks post-inoculation, horses in the dexamethasone group seroconverted 4 weeks post-inoculation, and 2 of the 3 controls seroconverted, 6 and 9 weeks after entering the study. All horses that received sporocysts tested positive for CSF antibodies at the end of the study, approximately 44 days post-inoculation and one of the two seropositive controls tested positive for CSF antibodies after 61 days in the study. The transport-stressed horses developed mild to moderate neurological signs 10-11 days after dosing that improved at least one grade by the final examination. The acclimated group developed mild neurological signs 9-16 days post-inoculation, and the signs did not change thereafter. The dexamethasone-treated horses developed mild neurological signs 9-16 days after dosing, and one horse from the group improved one neurological grade by the final examination. One control horse developed mild neurological signs 30 days prior to seroconversion. No attempts to recover *S. neurona* post mortem in neurological and other tissues were successful, although some microscopic inflammatory lesions were found in spinal cord and brainstem specimens.

In the second Ohio study (Sofaly et al., 2002), 24 seronegative weanling horses were subjected to the stress of transport from Saskatchewan, Canada to Columbus, Ohio and immediately dosed with sporocysts upon arrival at the research center. The strain of *S. neurona* used in the study was SN 37-R, derived from raccoon tissue fed to laboratory-reared opossums. Horses were divided into six groups of four horses, with one group serving as uninfected controls and the remaining five groups receiving 100, 1,000, 10,000 100,000 or 1,000,000 sporocysts in a single dose. No control horses seroconverted or

tested positive for CSF antibodies to *S. neurona*. Two of four horses that received 100 sporocysts produced serum antibodies 4-5 weeks post-inoculation. Horses that received 1,000 or 10,000 sporocysts seroconverted 3-4 weeks after dosing. One horse that received 10,000 sporocysts did not seroconvert. Horses that were given 100,000 sporocysts produced serum antibodies 3 weeks post inoculation, and horses receiving 1,000,000 sporocysts seroconverted 2 weeks post-inoculation. All seropositive horses had produced CSF antibodies to *S. neurona* when they were tested at days 28-35, except for two horses that received the 1,000 sporocyst dose. Neurological examinations by blinded evaluators revealed that horses in all groups developed mild to moderate neurological signs, but no dose-response trend of severity or time to onset (8-29 days) was detected. Additionally, one control developed neurological disease consistent with equine degenerative myelopathy (EDM) and one horse receiving 100,000 sporocysts did not develop neurological signs. Neurological signs were seen in 5 horses that tested negative for CSF antibodies to *S. neurona*. No attempt to recover or identify *S. neurona* post mortem was successful, although some horses in the study had microscopic inflammatory lesions suggestive of protozoan infection.

The most consistent findings of the experimental infection studies published to date (Fenger et al. 1997, Saville et al. 2001, Cutler et al. 2001, Sofaly et al., 2003) are that seroconversion appears to occur two to four weeks after horses receive a single dose of *S. neurona* sporocysts, neurological signs, when seen, are mild to moderate and do not appear to follow a dose-response relationship, and the parasite has yet to be identified or recovered in experimentally infected horses post mortem. All the studies suffered from low sample sizes. In the first study (Fenger et al., 1997), no two horses got the same dose

of sporocysts, although due to the fact that the inoculum was of mixed *Sarcocystis* species, this may not have made a difference in the interpretation of the results. In the other three studies (Saville et al. 2001, Cutler et al. 2001, Sofaly et al., 2003), there were no more than 4 horses per treatment group. This did not allow sufficient statistical power to detect differences between treatment groups with respect to days to seroconversion, onset of neurological signs or mean severity of neurological signs. Thus, while the data suggests that dexamethasone does not cause immunosuppression that increases susceptibility to EPM, there has not been a sufficiently powerful study to date that can demonstrate this with 95% confidence (Saville et al. 2001, Cutler et al. 2001). Likewise, in the study that evaluated sporocyst dose and neurological disease in transport-stressed horses, in which there were only four horses per group, it was not possible to determine whether sporocyst dose or CSF antibody status were associated with the severity of neurological signs (Sofaly et al., 2002). It is possible that horses that develop EPM from natural infections have some immunological characteristic that makes them different from the general horse population. That is, they may be more susceptible to infection or less able to clear the infection from the CNS than the experimental horses studied to date. At this time, a reliable equine disease model for EPM has yet to be established.

4. EPIDEMIOLOGY:

EPM is the most commonly diagnosed infectious equine neurological disease in the U.S (Dubey et al., 2001a). It has been reported in horses and ponies, but not in donkeys or mules (MacKay et al 1992; Dubey et al., 2001a). EPM usually occurs sporadically, and has not been shown to be transmissible from horse to horse (Dubey et

al., 2001a). Clusters of cases are unusual (Madigan and Higgins, 1987; MacKay et al., 1992). Rarely does one farm report more than one case, but some farms have had cases annually (Madigan and Higgins, 1987; MacKay et al., 1992), and there are two reports in the literature of clusters of cases on farms (Granstrom et al., 1992; Fenger et al., 1997b)

EPM is considered a New World disease. It has been diagnosed in horses in Canada, the United States, Panama and Brazil (Fayer et al., 1990; Granstrom et al., 1992; Masri et al., 1992). Cases reported in other parts of the world have been horses that originated from the United States (Fayer et al., 1990; MacKay et al., 1992; Katayama et al., 2003) or had traveled there prior to being diagnosed (Goehring and Sloet van Oldruitenborgh-Oosterbaan, 2001). EPM has been diagnosed in horses ranging in age from 2 months to 24 years (Fayer et al., 1990; MacKay et al., 1992). The possibility of transplacental transmission is suggested, given the fact that a 2-month-old foal was diagnosed by histopathology with EPM.

In a retrospective study of 364 histologically confirmed EPM cases from the U.S and Canada (Fayer et al., 1990), clinical cases were found to range in age from two months to greater than 19 years, but more than 60% of the cases were 4 years old or younger. No association was identified between the occurrence of EPM and the horses' geographic location or gender. There was also no apparent seasonal pattern to EPM. The breeds most commonly affected were Thoroughbred, Standardbred, and Quarter Horse, respectively. Other breeds in the study were: Appaloosa, Arabian, Morgan, Paint, Belgian and Crossbred.

In a smaller 1990 study of 82 EPM cases confirmed by histopathology (Boy et al., 1990), Standardbreds were overrepresented compared to the normal hospital population

at the University of Pennsylvania. In that study, 87.5% of the EPM cases were aged 6 or younger. Also, male horses appeared to be at greater risk of developing EPM. The authors acknowledged that there were associations within the data that may have confounded the results; young racehorses were most likely to be male at that hospital, and the mean age of the hospital population for the study period was not available for comparison.

In a case-control study of living horses in Ohio, putatively diagnosed with EPM, several factors were associated with the development of clinical disease (Saville et al., 2000). In comparison to controls, cases were at greater odds of being presented for diagnosis in spring, summer or fall, and cases were at greater odds of residing on operations where a previous case of EPM had occurred (Saville et al., 2000). Cases were at greater odds of being involved in racing or showing than controls, cases were at greater odds of having had a previous health event (such as lameness, injury or parturition) than controls, and cases were at greater odds of residing on operations where opossums were known to be present (Saville et al., 2000). In comparison to controls, cases were at lower odds of being 1 year of age or younger, being from an operation with a creek or river on the premises, or residing on an operation where all feeds and forages were secure from wildlife (Saville et al., 2000).

The incidence of clinical EPM cases appears to be low, despite the fact that antibody seroprevalence studies suggest many horses have been infected with *S. neurona* at some time in their lives. The incidence of EPM has been estimated to be 0.014% for the United States (N.A.H.M.S., 2001). In a Michigan seroprevalence study, 10 of 1121 (0.9%) horses had been diagnosed with clinical EPM within one year of participating in

the study, although none was considered an active case at the time the questionnaire was administered (Rossano et al., 2001). Thus, EPM can be characterized as a disease of high antibody seroprevalence and low clinical disease incidence.

S. neurona antibody seroprevalence studies have been performed on populations of presumably neurologically normal horses in Oregon (Blythe et al, 1997), Ohio (Saville et al., 1997), Michigan (Rossano et al., 2001), northern Colorado (Tillotson et al., 1999), and Chester County, Pennsylvania (Bentz et al., 1997). Serum antibody prevalence in Chester County, Pennsylvania was approximately 45.3% (Bentz et al., 1997). In Oregon, seroprevalence ranged from 22% to 65% from east to west, and overall prevalence was approximately 45% (Blythe et al, 1997). Seroprevalence was associated with geographic region; horses tested in the temperate, coastal regions were more likely to be infected than horses from the colder, arid eastern regions (Blythe et al, 1997). This difference was attributed to opossum prevalence and climate, since opossums were more abundant in the temperate climates (Blythe et al, 1997). In Michigan, seroprevalence followed a north-south gradient that also reflected the opossum distribution in the state (Rossano et al., 2001). There, 47% of the horses in the northernmost region were seropositive, 56% of the horses in the middle region were seropositive, and 62% of the horses in the southernmost region were seropositive (Rossano et al., 2001). Overall average statewide seroprevalence in Ohio was approximately 53.6%, and seroprevalence was negatively associated with days below freezing (Saville et al., 1997). This protective effect was hypothesized to be due to decreased sporocyst survival in cold conditions, as well as poor sarcocyst (tissue cyst) survival, causing a reduction of infections in the definitive host population. In northern Colorado, the antibody prevalence was 33.6%. There, a seasonal trend was

reported (Tillotson et al., 1999). Seroprevalence was higher among horses tested in spring, summer and fall than horses tested in winter. Two breed groups, warmbloods, and a category comprised of ponies and non-horse equids, had higher antibody prevalence than hot-blooded breeds and stock breeds. It was acknowledged by the authors, however, that the breed category variables might have indirectly accounted for other factors associated with the management of different types of equids. In that study, it was also speculated that there was an association between low opossum density and low antibody prevalence (Tillotson et al., 1999) In all of the cited studies, age was positively associated with seroprevalence (Bentz et al., 1997; Blythe et al, 1997; Saville et al., 1997; Tillotson et al., 1999; Rossano et al., 2001). No association has been identified between the risk of infection and horse gender (Bentz et al., 1997; Blythe et al, 1997; Saville et al., 1997; Tillotson et al., 1999; Rossano et al., 2001), or indoor versus outdoor housing Blythe et al., 1997; Rossano et al., 2001). In the Michigan study, which examined the most management factors of the studies to date, a mild association was found between seropositivity and access to pasture (Rossano et al., 2001). Feeding sweet feed was associated with decreased seropositivity, but this finding was questioned by the authors due to confounding with age and geographic region (Rossano et al., 2001). No association was found between farm size, animal gender, hay type or access to natural surface water and seropositivity (Rossano et al., 2001). Another analysis of the same study population did not reveal any herd-level management practices (applied to all horses on a given farm) and the proportion of animals seropositive (Rossano et al., 2002).

The prevalence studies published to date have been useful in quantifying *S. neurona* infection in normal horses, but they have suffered from notable limitations and

biases. For example, in Ohio (Saville et al., 1997) and northern Colorado (Tillotson et al., 1999), the sera used to determine antibody prevalence were obtained from equine infectious anemia (EIA) testing laboratories. In those studies, sample sizes were robust (1,056 equids in Ohio (Saville et al., 1997), 608 in northern Colorado (Tillotson et al., 1999), and to reduce bias, the researchers made random selections from the serum samples. However, the authors acknowledged that horses tested for EIA are apt to travel, and EIA samples were not necessarily representative of the actual horse populations of those geographic regions (Saville et al., 1997; Tillotson et al., 1999). Another limitation of using EIA samples is that only general descriptive data could be obtained about the horses tested. Although information regarding the location, age, breed and gender of the horse was recorded at testing (Saville et al., 1997; Tillotson et al., 1999), it was not possible to know how long the horse had been at the location where it was tested, and no horse management information was available for analysis. In Oregon, 334 horses were selected by a modified cluster-sampling technique (Blythe et al., 1997). The clusters were organized according to four distinct geographic regions, and veterinarians were recruited to systematically select neurologically normal horses from their practices for serum testing. No more than two horses from a given farm could be included in the study. Although this sampling technique may have resulted in a more representative sample of a region's horses than EIA testing samples, it was not a random selection process. In the Oregon study, information regarding individual horses and their housing was collected (Blythe et al., 1997). While this provided more detailed information, compared to EIA testing records, no information regarding feeds or watering systems was collected. Finally, the study of seroprevalence in Chester County, Pennsylvania was

performed on 117 serum samples collected from thoroughbred breeding farms (Bentz et al., 1997). Due to the small sample size this study was lacking in statistical power, and due to the small geographic area tested and the exclusion of all breeds but thoroughbred, the results have limited external validity beyond the study population. The Michigan study benefited from large sample size (98 farms, 1121 equids) and a stratified-random sample of farms on which all horses were tested, but the study was limited by the fact that the information on the opossum distribution in the state was 16 years old when the study was conducted (Rossano et al., 2001).

It is assumed that horses are infected with *S. neurona* by consuming hay, grain, pasture grass or water that has been contaminated with feces from the opossum (Dubey et al., 2001a). In other *Sarcocystis* species, the severity of infection has been related to the number of sporocysts fed (Urquhart et al., 1996), however the dose response is not known for *S. neurona* in equids. Since 1997, a number of experimental infection studies have been performed in which specific pathogen free horses were given doses of *S. neurona* sporocysts in an attempt to reproduce clinical EPM or document the life cycle of *S. neurona* (Fenger et al. 1997; Saville et al. 2001; Cutler et al. 2001; Cheadle et al. 2001a; Cheadle et al. 2001b). In the first study sporocyst doses were calculated, but were most likely sporocysts from opossums with mixed *Sarcocystis* spp. Infections (Fenger et al. 1997). In that study, although clinical neurological disease was produced by feeding large doses of sporocysts to foals, and inflammatory lesions consistent with EPM were found in three of the five experimental animals, at the end of the experiment no parasites were found by histopathology, and none could be cultured from the foals' neural tissue (Fenger et al. 1997). A recent study that utilized transport stress as a means of producing

immunosuppression in weanling horses reported that mild to moderate clinical signs were produced in horses who received sporocyst doses ranging from 100 to 1,000,000, with the most consistent signs seen in the 1,000,000-sporocyst group (Sofaly et al., 2002).

5. PATHOLOGY:

a.) GROSS PATHOLOGY:

S. neurona causes non-suppurative inflammation of the CNS, resulting in gross lesions in both gray and white matter and necrosis of affected neural tissue (Rooney et al., 1969; Mayhew et al., 1978; Fayer et al., 1990). The lesions are random in occurrence and vary in size from microscopic to several centimeters in diameter (Rooney et al., 1969; Mayhew et al., 1978; Fayer et al., 1990). Lesions are most common in the spinal cord or brain stem (Rooney et al., 1969; Mayhew et al., 1978; Fayer et al., 1990). When they are macroscopic, the lesion will appear discolored (pink, brown or yellow), the tissue may be soft and swollen, and it may have (multi-) focal hemorrhages (Rooney et al., 1969; Mayhew et al., 1978; Fayer et al., 1990). Treatment with anti-protozoal drugs is reported to decrease the likelihood that gross lesions will be detected upon postmortem examination (Dubey et al., 1991; Boy et al., 1990).

b.) HISTOPATHOLOGY:

The mechanism by which *S. neurona* gains access to the central nervous system has not been described, but the parasite has been identified in mononuclear cells, and occasionally giant cells, neutrophils and eosinophils (Rooney et al., 1969; Simpson and Mayhew, 1980; Dubey and Miller, 1986, Bowman et al., 1992). Perivascular cuffing

with inflammatory cells is evident in the affected areas and in the meninges that cover the lesions (Rooney et al., 1969; Mayhew et al., 1978). Demyelination of axons by macrophages has been reported, and degeneration of neurons and axons is commonly seen (Rooney et al., 1969; Bowman et al., 1992).

6. DIAGNOSIS:

A definitive diagnosis of EPM requires finding the parasite in neural tissue sections at postmortem examination (Mayhew et al., 1978; Hamir et al., 1993). Because the lesions can be microscopic in size, histopathology can be unrewarding (Hamir et al., 1993). In horses, the length of the spinal cord and size of the brain makes it difficult to adequately sample for lesions, even when clinical signs are used to localize the most likely area(s). Animals that have been treated with anti-protozoal drugs are even more difficult to diagnose by this method (Boy et al., 1990; Hamir et al., 1993). Immunohistochemical staining using polyclonal anti-*S. neurona* or anti-*S. cruzi* antibodies has been shown to increase success in locating the parasite, even in horses treated with sulfonamides (Hamir, et al., 1993). It is also possible to culture the parasite from neural tissue collected from horses suspected of having EPM (Davis et al., 1991; Bowman et al., 1992; Mansfield et al., 2001), but this process is elaborate and not normally done as part of a routine necropsy.

Antemortem diagnosis of EPM has evolved since the disease was first described. Because a variety of neurological signs may be present, clinical signs alone are not sufficient for accurate diagnosis of EPM (Mayhew and Greiner, 1986; Madigan and Higgins, 1987; Mackay et al., 1992; Furr et al., 2002).

The first diagnostic test available to veterinarians was an indirect fluorescent antibody (FIAX) titer performed on serum. The test measured reactivity to *Sarcocystis cruzi* bradyzoites from bovine cardiac tissue cysts, and was used to indicate the presence of *Sarcocystis* spp. infections, but was not specific to *S. neurona* (MacKay et al., 1997). In 1993, a western blot test that could detect antibodies to *S. neurona* in serum or cerebrospinal fluid (CSF) was developed (Granstrom et al., 1993). The western blot test used reactivity to proteins of 22, 13 and 10.5 kDa as the criteria for a positive test. These proteins were asserted to be specific to *S. neurona*, however the sensitivity and specificity of the test were not estimated. A subsequent western blot test that employed bovine-derived anti-*S. cruzi* antibodies to block cross-reacting proteins on blots was estimated to have sensitivity and specificity of $\leq$100% and 98%, respectively, for detecting antibodies to *S. neurona* in equine serum when simultaneous reactivity to the 30- and 16-kDa proteins was used as the positive criterion (Rossano et al., 2000).

A polymerase chain reaction (PCR) test has been offered since 1995 (Fenger et al., 1994). The advantage of the PCR test is that it can directly detect parasite DNA, rather than antibodies, in CSF (Fenger et al., 1994). The PCR test targeted the 18S small subunit ribosmal RNA (SSURNA) gene of *S. neurona* and related species, and production of a 484 base-pair product was the criteria for a positive test (Fenger et al., 1994; Fenger et al., 1995). Later investigations have shown that this portion of the target gene was not unique to *S. neurona*, and that the PCR test could not distinguish *S. neurona* from *S. falcatula* or *S. speeri*, but that *S. neurona* could be distinguished from the other species by using different primers and subjecting the 334-bp and 1,085-bp amplicons to restriction fragment length polymorphism enzyme digestion (Tanhauser et al., 1999).

Although it is a valuable research tool, in clinical use on CSF samples the PCR test appears to suffer from low sensitivity, making negative results unhelpful in a diagnosis (Dubey et al., 2001a). A positive result is strong evidence of active infection of the CNS with *S. neurona*, however (Dubey et al., 2001a). The apparently low sensitivity may be due to poor DNA extraction techniques in the laboratory, degradation of samples during shipping, or low numbers of *S. neurona* merozoites present in the small quantities of CSF that are usually submitted for testing. In one study, it was recommended that test sensitivity could be enhanced if veterinarians submitted CSF samples of greater than 5 ml, rather than the customary 1.5 ml (Marsh et al., 1996b). In 2001, a direct agglutination test using *S. neurona* merozoites was developed that was estimated to have 100% sensitivity and 90% specificity in experimentally infected mice (Lindsay and Dubey, 2001). Although the test has proved useful for wildlife antibody seroprevalence studies, it is not commercially available for use by equine practitioners as a diagnostic test.

At present, the western blot test is the most commonly used EPM test today (Dubey et al., 2001a). A positive serological test result indicates past or current infection with *S. neurona*, but does not confirm that the parasite has reached the central nervous system. Given the high estimated rate of serum antibody prevalence to *S. neurona* in clinically normal horses (>50% in some areas), testing of CSF is usually preferred (Fenger, 1997; MacKay, 1997; Furr et al. 2002). Clinical signs of multifocal neurological disease, accompanied by a positive CSF test allow for a reasonably reliable approach toward diagnosis, but do not constitute a definitive diagnosis of EPM (Fenger, 1997; MacKay, 1997; Furr et al. 2002). This is partly because IgG antibodies are only

indirect evidence of the presence of the parasite, and can remain after an infection has cleared. Also, false positive western blot results on CSF samples can be produced by the leakage of seropositive blood into the CSF, either at the time of collection or due to a disease process that causes leakage of serum proteins across the blood-brain barrier, such as EHV-1 encephalitis (Miller et al., 1999; Furr et. al 2002). In a recent analysis of paired serum and CSF western blot test results from horses being tested for diagnostic (not research) purposes, 29% of the horses that had serum antibodies tested negative for CSF antibodies (Rossano et al., 2003). In that study, the degree of blood contamination in the CSF samples was not known. Recent evidence suggests that horses with very high serum antibody titers to *S. neurona* could produce false-positive CSF tests, due to a minute amount of blood contamination (8 RBC/ul of CSF) (Miller et al., 1999) or by passive movement of serum antibodies across an intact blood-brain barrier (Furr, 2002). Fortunately, because *S. neurona* spends most of its time inside cells, not in the bloodstream (Dubey et al., 2001a), serum titers tend to be low, and high titers are not common (Duarte et al., 2003). It has been recommended that samples of CSF should have $\leq$ 50RBC/ul (Furr et al., 2002) to $\leq$ 100 RBC/ul (Daft et al., 2002) to be considered suitable for western blot testing.

A negative western blot test result of a CSF sample is generally accepted to be useful for ruling out EPM, due to the high sensitivity of the test (Furr et al., 2001a). One study based on histologically confirmed EPM cases supports this assumption, reporting the sensitivity of the western blot test on CSF to be 87% for horses with and 88% for horses without neurological abnormalities (Daft et al., 2002). In the same study, the specificity of the western blot was estimated to be 44% for horses with and 60% for

horses without neurological abnormalities. One weakness of the study, however, is that its definition of a "gold standard" negative was a horse in whom inflammatory lesions or parasite were not found in numerous CNS sampling points. Since it was not feasible to sample the entire brain and spinal cord of a horse, and histopathology is a technique of low sensitivity (Hamir et al., 1993), it is likely that there were horses in the study that were misclassified as gold standard negatives. This undoubtedly biased estimates toward lower specificity. Another study based on 48 samples from the same group of histologically confirmed cases found that a *S. neurona* indirect fluorescent antibody (IFA) test performed on serum was more accurate overall under receiver-operating characteristic analysis than the western blot on serum or CSF when a titer of 1:80 was classified as a positive result (Duarte et al., 2003). Again, the same weaknesses hold true in terms of the potential for misclassification of gold standard negatives, and now that a *S. neurona* vaccine has come to market, the value of serum IFA titers for diagnosis of EPM is questionable at this time.

Today, equine clinicians are encouraged to perform a thorough physical examination and order ancillary tests (such as radiographs and diagnostic tests for EPM and other diseases) to rule out musculoskeletal lameness and other neurological diseases before arriving at a diagnosis of EPM (Furr et al., 2002). This diagnosis should ideally be supported by clinical signs that are consistent with EPM and a positive western blot test result on uncontaminated CSF from the horse (Furr et al., 2002).

7. TREATMENT:

Drugs that interfere with folic acid synthesis in protozoa, by inhibiting dihydrofolate reductase/thymidylate synthase and dihydropteroate synthase, were the first to be employed in the treatment of EPM, and thus EPM was considered to be a treatable disease (Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987; Fenger, 1997; Mackay, 1997). Pyrimethamine and sulfa combinations were the standard therapy and it was recommended that treatment be given for 3 to 5 months (Fenger, 1997; Mackay, 1997). Relapses have been known to occur after this therapy, and treatment of these cases is usually less successful than it was during the treatment of the initial case (Fenger, 1997; Mackay, 1997). It has been estimated (but no published study supports this estimate) that approximately 70% of horses treated by this therapy respond favorably (Dubey et al., 2001a).

Prolonged antifolate therapy can result in undesirable side effects, including reduced spermatogenesis in stallions, possible harm to the fetus in pregnant mares, and bone marrow suppression in adult horses (MacKay, 1997). Blood counts may be used to monitor for evidence of toxicity due to the folic acid inhibitors (Dubey et al., 2001a). When leukopenia is detected in its early stages, lowering the dosages of the antifolates can prevent further progression (Mayhew and Greiner, 1986). Although oral supplementation of folic acid in horses treated for EPM has been recommended (Fenger et al., 1997), one study suggests that it may cause congential defects in foals born to dams treated with antifolates during pregnancy (Toribio et al., 1998). Folinic acid supplementation can reverse the folic acid inhibiting effect of the medication, but it is too costly for most equine applications (Mackay, 1997). Folinic acid can be provided to

horses at much lower cost in a diet of fresh green forage (Mackay, 1997; Toribio et al., 1998).

Since the late 1990's coccidiocidal drugs used in poultry species have been tested in clinical trials in horses. The first, diclazuril, had shown efficacy in treating acute *T. gondii* infections in mice (Lindsay et al., 1995). For reasons not made public, the drug has not yet been approved for use in horses. The second coccidiocide to enter clinical trials was ponazuril, which can be given to horses as a 15% oral paste by their owners (Furr et al., 2001). At present, it is the only drug labeled for the treatment of EPM. Ponazuril is given daily for 28 days, less time than the sulfa/pyrmethamine therapy requires (Furr et al., 2001). When the response to therapy in 101 horses was evaluated, 62% of the horses treated met the criteria for a successful treatment after 3 months of observation (Furr et al., 2001). (Horses who converted to a negative CSF antibody western blot result or who demonstrated an improvement of at least 1 neurological grade on a 0-5 scale were classified as treatment successes).

The final compound tested for EPM therapy is nitazoxanide (NTZ), a broad-spectrum anthelmintic used in human medicine, which is formulated as an oral paste for horses (Dubey et al., 2001a). The treatment course for NTZ was also 28 days. An early report based on 7 horses showed the efficacy of NTZ to be comparable in efficacy to the sulfa/pyrimethamine therapy (Vatistas et al., 1999). A subsequent study involving 70 horses reported that 63% of horses on the treatment improved one neurological grade or more or becoming negative for CSF antibodies on the western blot test, but the drug had a narrow margin of safety (McClure and DePalma, 1999). To date, NTZ has not been approved for use in horses.

In acute cases of EPM, anti-inflammatory drugs, such as phenylbutazone, dimethylsulfoxide and glucocorticosteroids may be used in conjunction with anti-protozoal drugs (MacKay, 1997). Corticosteroids used alone have been shown to hasten the progression of EPM in naturally infected horses (Mayhew et al., 1978; Mayhew and Greiner, 1986; Madigan and Higgins, 1987), but did not appear to affect neurological signs in experimentally infected horses (Cutler et al., 2001). In situations where EPM is thought to be related to a poor immune response in the horse, use of immunostimulants, such as levamisole, alpha-interferon, mycobacterial wall extract or killed *Propionibacterium acne*, have been recommended (MacKay, 1997). Vitamin E supplementation may help minimize damage from inflammation and aid healing of the central nervous system, although a standard equine diet of fresh or dried forage usually contains excess levels of vitamin E (MacKay et al., 1997).

8. PROGNOSIS:

In cases where EPM is treated with anti-protozoal drugs and the horse survives, no definitive post-mortem diagnosis is possible. Therefore, cure rates cannot accuately be determined. Horses that respond to treatment usually stabilize within a week (MacKay et al., 1992). Improvement may begin at that point and can continue for months or years following cessation of drug therapy (MacKay et al., 1992). Recovery is often incomplete, but if treatment begins soon after the onset of signs, the likelihood of a full recovery is greater (MacKay et al., 1992). Brain disease seems more likely to improve than spinal cord disease and muscle atrophy is most often permanent (MacKay et al., 1992).

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CHAPTER 2

DOES DAILY ADMINISTRATION OF PYRANTEL TARTRATE PREVENT INFECTION WITH *S. NEURONA* IN HORSES? AN EXPERIMENTAL CHALLENGE STUDY.

2.0 ABSTRACT:

Equine protozoal myeloencephalitis (EPM) is caused by the protozoan parasite *Sarcocystis neurona*. *In vitro* studies have shown that pyrantel tartrate is lethal to *S. neurona* merozoites. The purpose of this study was to determine whether daily administration of pyrantel tartrate prevents *S. neurona* infection in horses, as determined by the presence of antibodies in the serum. Twenty-four mixed breed weanling horses, confirmed free of infection by absence of serum antibodies to *S. neurona,* were group-housed at the Washington State University (WSU) Animal Resource Unit in outdoor pens and fed locally produced feeds. Horses were randomized into two groups: group A received pyrantel tartrate (Strongid C 2x) at the label dose and group B received a placebo pellet. Both groups were orally inoculated with 100 sporocysts per day for 28 days, 500 sporocysts per day for 28 days and 1,000 sporocysts per day for 56 days. Pyrantel tartrate or placebo was administered to the horses immediately following dosing with sporocysts. Blood was collected weekly and spinal fluid was collected monthly. Eleven adult horses from WSU that also tested negative for serum antibodies to *S. neurona* and were monitored as untreated, uninfected controls. Control horses were bled weekly on the same schedule as the experimental horses. All serum and CSF samples

were tested for antibodies to *S. neurona*. After 134 of sporocyst dosing, 10/12 horses in each treatment group were seropositive for antibodies to *S. neurona*. 4/12 horses in group A and 3/12 horses in group B had antibodies to *S. neurona* in CSF. No statistically significant difference was found between groups A and B for serum or spinal fluid tests (Fisher's exact test). Ten of 11 control horses remained seronegative. We conclude that daily administration of pyrantel tartrate does not prevent infection with *S. neurona* in horses when given according to label dose.

2.1 INTRODUCTION:

Equine protozoal myeloencephalitis (EPM) is a multi-focal neurological disease affecting horses and other equids of the Americas. Clinical signs of EPM may include weakness, ataxia, loss of proprioception, muscle wasting, behavioral changes, recumbancy and/or death (Dubey et al., 2001a; MacKay, 1997).

The most important etiologic agent of EPM is the protozoan parasite, *Sarcocystis neurona* (Dubey et al. 1991). *S. neurona* requires a definitive and intermediate host to complete its life cycle. Intermediate hosts of *S. neurona* identified to date include the domestic cat (Dubey et al., 2000), the nine-banded armadillo (Cheadle et al., 2001a), the striped skunk (Cheadle et al., 2001b), the raccoon (Dubey et al., 2001c) and the sea otter (Dubey et al., 2001b). Opossums serve as the only known definitive host of *S. neurona* (Dubey et al., 2001a). Horses and ponies are infected when they ingest sporocysts of *S. neurona* that are present in contaminated feed or water (Dubey et al., 2001a). To date, mature sarcocysts of *S. neurona* have not been detected in equids, therefore equids are considered to be dead end hosts (Dubey et al., 2001a). Recently, however, there was a report of a young colt with neurological disease in which both mature schizonts in the brain and spinal cord and mature sarcocysts in the tongue were found (Mullany et al., 2003). Electron microscopy revealed that the sarcocysts had characteristics that were consistent with published characteristics of *S. neurona.* Testing for genetic markers showed that the sarcocysts produced *Sarcocystis*-specific PCR products and these products exhibited banding patterns characteristic of *S. neurona.* Further studies will be required to determine whether horses are true intermediate hosts to *S .neurona* or if this

colt was abnormally susceptible due to his young age, or some form of immunosuppression.

S. neurona can infect the central nervous system (CNS) of equids, which may lead to the development of neurological disease. The U.S. Department of Agriculture estimated the incidence of EPM in 1998 to range from 0.06% in the southern U.S. to 0.43 in the central U.S., with a national average of 0.014% (N.A.H.M.S.). Cross-sectional studies conducted in several geographic areas of the United States revealed that in regions of western Oregon and southern Michigan, seroprevalence was as high as 60-65% (Blythe, et al., 1997; Rossano et al., 2001) and as low as 22-34% in regions of eastern Oregon and northern Colorado (Blythe et al., 1997; Tillstone et al., 1999). Taken together, the disease incidence and antibody seroprevalence data suggest that many horses are infected with *S. neurona* at some time in their lives, but very few of them develop clinical disease. Despite the low incidence of EPM, there is great interest among members of the horse industry to find effective preventive strategies. This interest to develop effective strategies can be attributed to the potential loss of use of the horse and the high cost of treating the disease. In 2001, a whole-cell killed vaccine of *S. neurona* merozoites was granted a conditional license as an aid for the prevention of EPM (Fort Dodge Animal Health) but the efficacy of the vaccine in horses has yet to be established. An important drawback of the vaccine is that vaccinated animals will test positive for serum antibodies, thus confounding the diagnosis of neurological disease in vaccinated horses.

The drug pyrantel tartrate has shown promise as a preventive agent in *in vitro* studies against the merozoite and sporozoite stages of *S. neurona* (Kruttlin et al., 2001;

Kruttlin et al., 2003). In those studies, the drug was lethal to the parasite when exposed to concentrations higher than 0.005M. In contrast, a study using gamma-interferon knockout mice, the drug was not effective in preventing infection with *S. neurona* at a dose of 4-5 mg pyrantel tartrate per mouse, however the drug may have been inactivated by being kept in a water solution for long periods of time (Lindsay and Dubey, 2001). Because of the encouraging results of Kruttlin et al. (2001 and 2003) it was decided to evaluate the efficacy of this drug in preventing infection in horses. Therefore, the purpose of this study was to test whether daily pyrantel tartrate (Strongid C 2x) could prevent infection by *S. neurona* in horses. The specific hypothesis tested in this study was that daily administration of pyrantel tartrate prevents infection with *S. neurona* in horses when given at the label dose.

2.2 MATERIALS AND METHODS:

2.2.1 The parasite:

S. neurona sporocysts were produced using a specific-pathogen-free laboratory-reared opossum. The opossum was fed muscle tissue from a raccoon that had been experimentally infected with *S. neurona* strain SN-37R (Sofaly et al., 2002), which was kindly provided by J.P. Dubey. At day 14 post-infection, the opossum was euthanized using CO_2 asphyxiation and the small intestine was removed, flushed with saline solution, and stripped of its mucosa. Sporocysts were harvested from the resulting tissue slurry according to previously described methods (Murphy and Mansfield, 1999) and stored in Hank's balanced salt solution with amikacin sulfate (100 μg/ml), penicillin G

(100U/ml) and amphotericin B (1.25 μg/ml) in a refrigerator for 1 year prior to the start of the study. Sporocysts were confirmed to be *S. neurona* by PCR with restriction fragment length polymorphism (RFLP) digestion (Tanhauser et al., 1999). Before being administered to animals, the sporocysts were further purified of cell debris and bacteria using a previously described potassium bromide gradient separation technique (Elsheikha et al., 2003.).

2.2.2 Verification of sporocyst viability:

Two months before the study, 3 female gamma-interferon knockout mice (Jackson Laboratories, Strain Name: C.129S7(B6)-*Ifng*^{tm1Ts}) were inoculated with subsamples of SN-37R sporocysts. Two mice received 1,000 sporocysts and 1 mouse received 1,500 sporocysts. The doses were gavaged to the stomach in sterile water solution using a 5 French rubber catheter. The mice were provided free choice food and water and were observed daily for general health and development of neurological signs. When neurological disease was observed, the mice were euthanized using CO_2 asphyxiation and homogenized samples of brain tissue were placed on equine dermal cell culture and observed weekly for growth of *S. neurona*. Merozoites harvested from the cell cultures were confirmed to be *S. neurona* using PCR with RFLP (Tanhauser et al. 1999). Mice were also submitted for histopathologic examination to confirm protozoal encephalitis.

Thirty-five days after initiation of the study, viability of the sporocysts used in the inoculum given to horses was confirmed again in 3 female γ-IFN knockout mice (Jackson Laboratories, Strain Name: C.129S7(B6)-*Ifng*^{tm1Ts}). On this occasion, 2 mice received

100 sporocysts and 1 mouse received 1,000 sporocysts in sterile water using a 5 French rubber catheter. Mice were euthanized as mentioned above when neurological signs were apparent, and brain tissue was harvested and placed on equine dermal cell culture and observed for parasite growth. Once again, mice were submitted for histopathologic examination and confirmed to have protozoal encephalitis.

2.2.3 The horses:

Twenty-four mixed breed weanlings, approximately 5 months old at the start of the study, were obtained from an equine ranch in North Dakota. The horses were delivered to the Animal Research Unit at Washington State University (WSU), in Pullman, Washington and allowed to acclimate in isolation on pasture for 2 weeks. Throughout the horses were fed alfalfa and grass hays grown in eastern and central Washington, an arid region where opossums are not reported to live (Figure 2.1.). Some locally produced sweet feed was provided to the horses as a training motivator. The horses were subsequently group-housed in adjacent outdoor paddocks with sparse vegetation, according to their treatment group, with access to shelter and free choice hay and water. A woven wire fence with an electrified top wire separated the two pens, limiting, but not excluding, horse-to-horse contact. (A diagram of the paddock layout is in Appendix I.) Eleven adult horses, maintained at WSU for teaching and research, were also studied as untreated, uninfected controls to insure that horses maintained on the same feeds as the experimental horses did not become infected with *S. neurona*. Five of the controls were housed in a paddock directly adjacent to the experimental horses, 6 were housed at another horse facility on the same campus, approximately 2 miles away.

Prior to arrival at WSU, the 24 experimental horses were dewormed with moxidectin and vaccinated against eastern and western encephalitis, tetanus, influenza and equine herpes virus I. They were dewormed again with ivermectin at the start of the study and booster vaccines were administered approximately 1 month after the first vaccine (10 days prior to the start of sporocyst dosing). West Nile Virus vaccines were given at days 10 and 35 after the start of sporocyst dosing.

2.2.4 Sporocyst inoculation:

Sporocysts were counted for the stock solution using a hemacytometer. In order to obtain the most accurate estimate possible, this was accomplished by keeping the solution constantly moving with stirring and by determining the mean of 32 counts.

The sporocyst inoculum for the horses was prepared daily, immediately prior to dosing. Sporocysts from the stock solution were suspended at the desired dose in a 70% corn syrup and 30% water mixture with stirring. A 2 ml dose was delivered to the horses using an oral syringe.

The goal of the dosing scheme was to mimic a low-dose, chronic infection situation and use the minimum number of sporocysts needed to achieve seroconversion. The minimum number of SN-37R sporocysts used to produce seroconversion in experimentally infected horses subjected to transport stress was reported to be a single dose of 100 (Sofaly et al., 2002). Thus, 100 sporocysts/day was chosen as the dose for this study. For the first 28 days horses were given 100 sporocysts/day. When weekly western blot testing showed no seroconversion, the dose was increased to 500 sporocysts/day for an additional 28 days. When that dose failed to produce

seroconversion, the horses were given 1,000 sporocysts/day for 56 days, after seroconversion had been detected in some horses at day 14 of this dose.

2.2.5 Biosecurity

Efforts were made to prevent the spread of sporocysts from experimental horses to the on-farm controls and other horses at the Animal Resources Unit. Foot baths were placed outside the experimental pens, and persons exiting the pens used them to wash contaminants from their shoes or boots. When experimental horses were fed, their hay was thrown into the rack from the adjacent pen, thus preventing daily truck traffic in the pens. Also, the experimental pens were the last ones cleaned on the farm, and the equipment was immediately hosed afterwards (The pen layout and cleaning protocol is shown in Appendix II).

2.2.5 Pyrantel tartrate treatment and placebo:

Upon arrival at the study site, the horses were randomized into treatment groups A and B. Group A received Strongid® C 2x (Pfizer, Inc) and group B received a look-alike placebo pellet provided by Pfizer, Inc. The two treatments were put into buckets marked A or B prior to shipment to WSU, and all persons associated with the daily conduct of the study were blinded to the identity of the treatments.

Treatments were administered to the horses daily, starting 4 days prior to the start of sporocyst dosing and continued until the last day of the study. When both sporocysts and treatments were given, the sporocysts were administered first, immediately followed by the treatment. Doses of treatments were determined according to the weight of each

horse, with each horse receiving 1 oz of pellets per 250 lbs of body weight. Horses were weighed monthly and doses were adjusted as they grew. Doses were measured using the cup provided with the Strongid® C 2x. Pellets were delivered orally in a 60cc syringe with the end cut off, then the horse was observed while it chewed and swallowed the pellets. More pellets were given if a horse dropped any. If a horse was reluctant to eat the pellets, a small amount of corn syrup was squeezed into the syringe to improve palatability. The complete protocol used for horse dosing is presented in Appendix III.

2.2.6 Physical assessment:

Horses were examined for neurological deficits, conformational defects and any gait abnormalities on day 0 of the study. These examinations were performed loose, as the horses were not yet trained to lead. Subsequent examinations took place in hand 2 weeks later, then on a weekly basis thereafter until the end of the study. These physical examinations were made by investigators who were blind to identity of the treatments the horses were receiving and results were recorded on a neurological evaluation form (Appendix IV). Briefly, the horses were assessed for cranial nerve deficits, abnormal stances and their ability to circle, back and trot in both directions. Horses were also observed daily for general health when treatments were being administered.

2.2.7 Diagnostic tests:

Serum and cerebrospinal fluid (CSF) were tested for antibodies against *S. neurona* using the western blot test offered by the Diagnostic Center for Population and Animal Health at Michigan State University (MSU). The test was performed using previously

described methods (Rossano et al. 2000) and antibody reactivity to both the 30- and 16-kDa antigens was the criterion for a positive test result. Serum and CSF samples were stored in a refrigerator and tested as soon as possible upon arriving at the laboratory. They were not frozen for long-term storage until after a final western blot result had been obtained, approximately one month in most cases.

2.2.8 Specimen collection:

Blood samples were collected from experimental and control horses by jugular venipuncture on day 0 and weekly thereafter until the conclusion of the study. The serum fraction was removed from the blood clot, stored in a refrigerator, and shipped with cold packs overnight to MSU for testing.

CSF was first collected from the 24 experimental horses on days 11 and 12 of the study, then approximately monthly thereafter. There were 5 collections in total. The CSF collections from the horses were performed over 2 days (12 horses per day), starting with group A the first month, group B the second month and alternating groups thereafter. CSF was collected from the atlanto-occipital site while the horses were under general anesthesia. Horses were kept in stalls for observation for several hours following the procedure, then returned to their group pens at the end of the day. The complete protocol for CSF collection is presented in Appendix V.

Samples of CSF in EDTA were immediately submitted to the Clinical Pathology Laboratory at WSU for cytological evaluation. Red blood cells and nucleated cells were counted and any cytological abnormalities were reported. In order to assure that positive

CSF antibody test results were not false-positives due to blood contamination, samples exceeding 50 RBC/ul were excluded from the analysis (Furr et al., 2002).

Feces were collected from each horse at the time of CSF collection. Three-gram samples of feces from each sample were tested by sucrose flotation for the presence of unexcysted sporocysts.

Four horses were euthanized, using pentobarbitone sodium (Beuthanasia-D, Schering Animal Health) to effect, at the end of the study and necropsies were performed. Three showed no neurological abnormalities, but were selected because they were deemed unadoptable due to conformational defects. One of the 4 horses was selected because it developed neurological disease that coincided with the development of serum antibodies to *S. neurona*. Neurological tissues (brain, brain stem and cervical, thoracic and lumbar sections of spinal cord) were collected and shipped overnight to MSU to attempt to culture *S. neurona* using a previously published technique (Mansfield et al., 2001). Samples of the same tissues also were fixed in formalin and examined for histopathologic evidence of EPM. Additionally, fresh and formalin-fixed samples of tongue, diaphragm, upper thigh muscle, masseter muscle, heart, liver, small intestine and lung were collected and shipped to MSU for histopathologic examination. The complete protocol for the collection of tissues is presented in Appendix VI.

2.2.9 Statistical analysis of results:

Test results for each horse were recorded individually in a spreadsheet. The numbers of animals testing positive and negative by the Western blot test at the end of the study were compared by treatment group for serum and CSF samples in 2 x 2 tables using

the Fisher's exact test, two-tailed (SAS). The Fisher's exact test was used because it is an appropriate test for discrete data, and low expected values in some cells made the chi-square test an inappropriate choice.

The days to seroconversion for serum results also were compared by treatment group and gender, using the Cox Proportional Hazards model (EGRET). Due to the small number of observations, treatment and gender were examined separately in univariable models. The Cox Proportional Hazards model was selected for this analysis because there were horses in the study that did not seroconvert, so in order to preserve their contribution to the results they were classified as "right censored" observations in the models.

In all analyses, a p-value of ≤ 0.05 was deemed significant.

2.3 RESULTS:

Seroconversions were initially detected in two horses (one from group A and one from group B) on day 77 (day 21 of receiving the 1,000 sporocysts/day dose). At the conclusion of the study, there were 10/12 horses in each group that were seropositive. There was no statistically significant difference between groups in the number of horses that were seropositive ($P \leq 1.00$). There was also no significant difference in days to seroconversion between treatment groups ($P \leq 0.71$) or by gender ($P \leq 0.21$). All on-farm control horses remained seronegative throughout the study. One off-farm control horse developed a positive serum western blot test on day 35 of the study, and continued to test positive thereafter. The other 5 off-farm control horses remained seronegative

throughout the study. A summary of the course of seroconversions by treatment group can be viewed in Table 2.1.

The first positive CSF western blot antibody test was detected at the 4th sampling date (104-105 days since the beginning of the study). At the 5th and final sampling date (137-140 days since the beginning of the study) 4/12 horses in group A tested positive for CSF antibodies and 3/12 horses in group B tested positive. There was no statistically significant difference between groups ($P \leq 1.00$).

At the last CSF collection, which was used for the final analysis, red blood cell counts ranged from 0 to 29 (mean = 2.58, median = 0) for group A and from 0 to 2 (mean = 0.25, median = 0) for group B. All CSF samples were below 50 RBC/ul and were, therefore, included in the data analysis. Nucleated cell counts ranged from 0 to 5 for group A (mean = 1.83, median = 1.5) and from 0 to 5 for group B (mean = 1.58, median = 1). No cytological abnormalities were seen. A complete summary of blood contamination from all CSF collections during the study is shown in Table 2.2.

No sporocysts were found in fecal samples collected from experimental horses during the course of the study.

Neurological examinations revealed one colt to be a probable subtle wobbler on day 49 of the study, 42 days before seroconversion. Its condition did not change appreciably thereafter. One filly (#27) developed mild asymmetric neurological signs that coincided with its seroconversion. It was selected for necropsy. No other horses in the study exhibited neurological signs during the study.

Postmortem examination of the 4 horses chosen for necropsy did not reveal any gross CNS lesions. *S. neurona* was not cultured from brain, brain stem or spinal cord

from any of the horses. One myofiber in the section of upper thigh muscle from horse #27 contained one protozoal cyst suggestive of *Sarcocystis* species, but further investigation of adjacent tissue did not reveal any additional cysts. Lack of material precluded additional work to identify the species of the cyst. No CNS lesions were found in horse #27. In a section of tongue from horse #154, a small, discrete aggregate of lymphocytes, macrophages and plasma cells was found in deep muscle but no protozoal organisms were identified. A longitudinal section of cervical spinal cord from horse #154 showed one thin, linear area of Wallerian degeneration. No significant histological findings were seen in a cross-section of cervical spinal cord taken from the same area, and no lesions were seen in any other tissue from that horse. No histopathologic abnormalities were found in the tissues of the remaining 2 horses that were necropsied (#33 and #42). All of the horses that were examined were seropositive, however, only horse #154 tested positive for CSF antibodies at the final collection. A summary of post-mortem findings and western blot test results are presented in Table 2.3.

2.4 DISCUSSION:

The results of this study clearly demonstrate that when given according to the label dose for Strongid C 2X, daily pyrantel tartrate did not prevent or delay infection of the experimental horses with *S. neurona*, as reflected by the production of antibodies in the serum. In addition, there was no significant difference between treatment groups when the proportion of CSF-positive horses were compared, suggesting that pyrantel tartrate did not have an effect on the ability of the parasite to gain access to the central nervous system. Horse gender was also not a significant factor in time to seroconversion,

although gender was a stronger association than treatment group, with males being the group that seroconverted sooner.

The fact that all of the on-farm control horses and 5/6 of the off-farm control horses remained seronegative suggests that the experimental horses seroconverted as a result of the sporocyst doses they were given not from sporocysts in feed from central and eastern Washington. The off-farm control horse that tested positive, did so 42 days before any experimental horse did, during which time they received daily doses of *S. neurona*. The horse, an aged Quarter Horse gelding, had lived in Colorado as recently as 1999, and had been donated to WSU in June of 2000 because he was narcoleptic. No other history was available on the horse, such as other places it may have resided in its lifetime. It is possible that the horse had an existing latent or subclinical infection that had been reactivated to the extent that he produced antibodies in response. In following naturally infected horses in Michigan, our research group has observed horses convert from seronegative to seropositive back to seronegative status (Chapter 3 of this dissertation). Perhaps the off-farm control horse exhibited a similar pattern of antibody waxing and waning.

The difficulty we experienced in producing seroconversion in these horses was markedly different from the experience of other researchers who used the same strain of *S. neurona*. In a study that utilized long distance transport as a stressor prior to experimental infection of horses, seroconversion was achieved using doses as low as 100 sporocysts (Sofaly et al., 2002). In the present study, daily doses of 100 sporocysts/day for 28 days and daily doses of 500 sporocysts/day for 28 days failed to induce seroconversion in any of the 24 experimental horses. It was not until 21 days after

receiving 1,000 sporocysts/day that the first seroconversions were seen. The disparity in results in these studies could be due to a number of factors. The immunosuppression induced by long distance transport may have made the horses in the previous study (Sofaly et al., 2002) more susceptible to infection, or the horses may have had minimal gut fill when they were dosed with sporocysts upon arrival at the study site. In contrast, the horses in the present study were given over 2 weeks to recover from transport and acclimate to their new surroundings before beginning the study, and, thus, were probably not immunosuppressed when sporocyst dosing began. They also were given free choice hay, and most likely had consistent gut fill during the study. This may have hindered the ability of *S. neurona* sporozoites to reach and penetrate the intestinal epithelial cells of the horses, due to physical barriers and the constant flow of ingesta. Another possible explanation for the difference in results is that in the previous study (Sofaly et al., 2002), horses were dosed with nasogastric tubes and the horses in the present study were dosed with oral syringes. While the horses dosed by nasogastric tubes were more likely to receive the full dose of sporocysts intended, it is unlikely that this difference would not have been overcome by the fact that the horses in the present study were dosed on a daily basis for a long period of time before seroconversion occurred. Finally, the previous study (Sofaly et al., 2002) may have had greater success in infecting horses because their sporocysts were fresher than those used in the present study, which had been stored for 1 year in Hank's balanced salt solution. While it is not possible to make a direct comparison between inoculums to determine this, the pathogenicity of the sporocysts used in the present study was demonstrated by the fact that they produced neurological

disease in gamma-interferon knock-out mice at 1 tenth the standard infective mouse dose of 1,000 sporocysts (Dubey et al., 2001d).

The fact that *S. neurona* was not cultured or identified in experimental horses at postmortem examination was not surprising, considering that only 1 of the 4 horses selected was found to have CSF antibodies to *S. neurona* at the time of euthanasia. Other studies, in which larger doses of sporocysts were used, have failed to recover *S. neurona* post mortem from experimentally infected horses (Fenger et al., 1997; Cutler et al., 2001; Saville et al., 2001; Sofaly et al., 2002).

The proportion of seropositive horses in the present study that also tested positive for CSF antibodies was 29% (7/24). In a recent study of 181 paired diagnostic samples from horses suspected of having EPM, 71% of the seropositive horses had CSF antibodies to *S. neurona* (Rossano et al., 2003). This difference in proportions could be explained by the fact that the diagnostic samples came from horses more likely to have active central nervous system infections with *S. neurona*, as opposed to the mostly neurologically normal study population described here, and that in the present study, no CSF sample exceeded the recommended limit of 50 RBC/ul (Furr et al, 2002), thus the chance of false positive test results due to blood contamination was low. Information regarding blood contamination of the clinical samples was not available, but it is likely that some samples were contaminated, thus biasing the results (Rossano et al., 2003). The success seen in the present study with CSF collection from the atlanto-occipital site demonstrates the advantage of using this method when general anesthesia is deemed safe. Only 4/120 (3.3%) of CSF samples collected this way were unusable due to blood contamination.

The response of the immunocompetent experimental horses to low, daily dosing of sporocysts suggests that the high antibody seroprevalence seen in U.S. horses may be the result of a different exposure scenario. Perhaps horses receive larger doses of sporocysts under natural conditions by consuming opossum feces directly, rather than via low-level contamination of feed or water. Perhaps horses are made vulnerable by certain circumstances, such as an immunosuppressive event or fasting to the point of having an empty gut when sporocysts are ingested. Further studies will be required to determine the most likely conditions under which horses are naturally infected with *S. neurona*.

2.5 CONCLUSIONS:

Daily administration of pyrantel tartrate does not prevent or delay infection of horses with *S. neurona*, and male horses did not seroconvert significantly faster than females when given low daily doses of sporocysts.

Table 2.1. Summary of seroconversion for experimental and control horses. NC = no seroconversion

Group A Pyrantel Tartrate			Group B Placebo Pellet			Group C Uninfected Untreated Controls		
Horse ID	Days to 1st positive test	Days to 1st positive test at 1,000 sporocysts/day	Horse ID	Days to 1st positive test	Days to 1st positive test at 1,000 sporocysts/day	Horse ID	Days to 1st positive test	Days to 1st positive test at 1,000 sporocysts/day
5	NC	-	**7**	104	48	**58**	35	-
24	NC	-	**34**	104	48	**123**	NC	-
27	104	48	**35**	113	57	**141**	NC	-
33	113	57	**37**	113	57	**144**	NC	-
36	104	48	**39**	NC	-	**150**	NC	-
40	104	48	**41**	NC	-	**156**	NC	-
42	98	42	**59**	98	42	**157**	NC	-
43	98	42	**105**	98	42	**161**	NC	-
64	91	35	**106**	98	42	**165**	NC	-
107	104	48	**154**	104	48	**180**	NC	-
160	77	21	**163**	77	21	**184**	NC	-
173	98	42	**195**	113	57			

Table 2.2. Summary of red and white blood cell counts from all CSF collections.

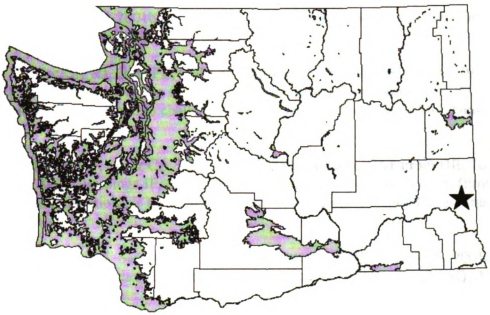
Variable	1st Collection	2nd Collection	3rd Collection	4th Collection	5th collection
RBC/ul range	0-4	0-1512	0-6750	0-20	0-29
RBC/ul median	0	0	0-1	0	0
WBC/ul range	0-6	0-4	0-14	0-5	0-5
WBC/ul median	1	0	1	1	1
# Positive WB tests	0/24	0/24	0/24	1/24	7/24
# > 50 RBC/ul	0	3	1	0	0

Table 2.3. Postmortem examination results for the four horses euthanized at the end of the study. Only horse #27 exhibited neurological signs, grade 2.0-2.5.

Horse ID number	Pathological findings	Final western blot antibody test – serum	Final western blot antibody test - CSF
27	One sarcocyst of undefined species in thigh muscle	Positive	Negative
33	None	Positive	Negative
42	None	Positive	Negative
154	One thin area of Wallerian degeneration in cervical spinal cord	Positive	Positive

Figure 2.1 Washington Gap Analysis Project's Predicted Distribution Map published by the University of Washington depicting the opossum distribution in the state. Opossums were not reported to be in the southeast portion of the state, where the study was conducted.

Virginia Opossum *(Didelphis virginiana)*



Legend:

★ = Study site

= Core Habitat

- **Breeding Range Map**
  The green area shows the predicted habitats for breeding **only**. The habitats were identified using 1991 satellite imagery, other datasets and experts throughout the state, as part of the Washington Gap Analysis Project.

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CHAPTER 3

DOES DAILY ADMINSTRATION OF PYRANTEL TARTRATE PREVENT *S. NEURONA* INFECTION IN HORSES? A CONTROLLED FIELD INTERVENTION STUDY.

3.0 ABSTRACT:

Equine protozoal myeloencephalitis (EPM) is primarily caused by the protozoan parasite *Sarcocystis neurona*. *In vitro* studies have shown that pyrantel tartrate is lethal to *S. neurona* merozoites and sporozoites. We hypothesized that daily administration of pyrantel tartrate prevents *S. neurona* infection in horses, as determined by the presence of antibodies in the serum. This hypothesis was tested by conducting a double-blind controlled field intervention study on three Michigan horse farms. Thirty-four yearling horses were identified as either seronegative or suspect positive by the western blot antibody test for *S. neurona*. These horses were randomized into two groups: group A received pyrantel tartrate (Strongid C 2x) at the label dose and group B received a placebo pellet. Pellets were fed daily, mixed with grain by farm staff according to label instructions for Strongid C 2x. After receiving treatments for approximately 2 weeks, the horses were tested again for serum antibodies to finalize entry into the study. After this final screening test, 11 horses on 2 farms were available for follow-up. One of these horses was subsequently sold. Horses who completed the study were tested for serum antibodies approximately bi-monthly. Test results for each treatment group were summarized in a 2 x 2 table and tested for independence by the two-tailed Fisher's exact test.

Six months later, at the conclusion of the study, 1/3 horses receiving daily pyrantel tartrate (group A) was seronegative and 2/4 horses receiving the placebo (group B) were seronegative. No statistically significant difference was found between groups A and B for serum antibody tests ($P < 1.00$). We conclude that daily administration of pyrantel tartrate does not prevent infection with *S. neurona* in horses when given according to label instructions under field conditions.

3.1 INTRODUCTION:

Equine protozoal myeloencephalitis (EPM) is a potentially serious neurological disease affecting horses and ponies of the Americas. Clinical signs of EPM may include weakness, incoordination, loss of proprioception, behavioral changes, muscle wasting, recumbancy and/or death (Dubey et al., 2001a; MacKay, 1997).

The primary causative agent of EPM is the protozoan parasite, *Sarcocystis neurona* (Dubey et al. 1991). *S. neurona* requires a definitive and intermediate host to complete its life cycle. Opossums serve as the only known definitive host of *S. neurona* (Dubey et al., 2001a). Intermediate hosts of *S. neurona* identified to date include the domestic cat (Dubey et al., 2000), the nine-banded armadillo (Cheadle et al., 2001a), the striped skunk (Cheadle et al., 2001b), the raccoon (Dubey et al., 2001c) and the sea otter (Dubey et al., 2001b). Horses and ponies are infected when they ingest sporocysts of *S. neurona* that are present in feed or water that have been contaminated with opossum feces (Dubey et al., 2001a). To date, no published accounts of mature sarcocysts of *S. neurona* detected in equids exist, therefore equids are generally considered to be dead end hosts (Dubey et al., 2001a). Recently, however, there was a report at the 2003 annual meeting of the American Association of Veterinary Parasitologists of a young colt with neurological disease in which both mature schizonts in the brain and spinal cord and mature sarcocysts in the tongue were found (Mullany et al., 2003). Electron microscopy revealed that the sarcocysts had features identical to published features of *S. neurona.* Testing by PCR with RFLP showed that the sarcocysts produced *Sarcocystis* specific PCR products and these products exhibited banding patterns characteristic of *S. neurona.* Further studies will be required to determine whether horses are true intermediate hosts to

S .neurona or if this colt was uniquely susceptible due to his young age or some form of immunosuppression.

Once *S. neurona* invades the central nervous system (CNS) of an equid, neurological disease may develop. The U.S. Department of Agriculture estimated the annual incidence of EPM cases in 1998 to be 0.014%, or 14 cases per 1000 horses (N.A.H.M.S.). Cross-sectional studies conducted in various geographic areas of the United States reported that in regions of western Oregon and southern Michigan, seroprevalence was as high as 63-65% (Blythe, et al., 1997; Rossano et al., 2001) and as low as 22-34% in regions of eastern Oregon and northern Colorado (Blythe et al., 1997; Tillstone et al., 1999). Collectively, these data suggest that many horses are infected with *S. neurona* at some time in their lives, but few of them go on to develop EPM. Despite the low incidence of EPM, there is great interest among members of the horse industry to find effective preventive strategies. This great interest for effective preventive strategies can be attributed to the high cost of treating the disease, and the poor recovery seen in some treated horses. In 2001, a whole-cell killed vaccine of *S. neurona* merozoites was granted a conditional license for the prevention of EPM (Fort Dodge Animal Health). The efficacy of the vaccine in horses has yet to be demonstrated. One disadvantage of using the vaccine is that vaccinated animals will test positive for antibodies to *S. neurona*, thus confusing the diagnosis of vaccinated horses presenting neurological signs.

The drug pyrantel tartrate has shown promise as a preventive agent in an *in vitro* study against the merozoite stage of *S. neurona* (Kruttlin et al., 2001). In that study, the drug was lethal to the parasite at concentrations greater than 0.0025M ($8.91X10^{-4}$ g/ml). In another *in vitro* study, not yet published but presented at the 2003 annual meeting of

the American Association of Veterinary Parasitologists, pyrantel tartrate was tested on the sporozoite state of the parasite (which is the stage of *S. neurona* that infects horses intestines and would be most likely to be exposed to the drug). There, the drug was found to be effective at concentrations of 0.005M or greater. Based on these encouraging results, we hypothesized daily administration of pyrantel tartrate (Strongid C 2x®) could prevent infection by *S. neurona* in horses when used by horse farm operators according to label instructions. The purpose of this study was to conduct a field intervention trial to test the hypothesis.

3.2 MATERIALS AND METHODS:

3.2.1 Study design:

The study was a double-blind field intervention trial. Persons administering the treatments and the person analyzing the data were "blinded" to the identity of the treatment groups. When preliminary results were given to the primary investigators who knew the treatment identities, the treatments were described by yet another set of names in order to prevent bias in the interpretation of results by the principal investigators.

3.2.2 Study population:

The study focused on yearling horses in order to have the greatest chance to enroll a population of seronegative animals. Horses could not have been vaccinated for EPM to be enrolled in the study or while participating in the study.

3.2.3 Sample size calculations:

A seroprevalence study conducted in Michigan revealed that 48% of horses in that state tested positive for antibodies to *S. neurona* by the time they were yearlings (Rossano et al., 2001). Therefore, it was expected that at least 50% of the horses receiving the placebo would be seropositive by the end of the observation period. Assuming 95% significance ($p<0.05$) and 80% power, two-tailed hypothesis testing, and 50% rate of seroconversion among horses on the placebo, it was determined that there should be at least 55 foals in each treatment group to detect a risk ratio of at least 1.5 for horses on the placebo (Thrusfield, 1995). We proposed to enroll 60 foals in each cohort, to accommodate for potential losses to follow-up, thus we attempted to total of 120 horses in the study. Due to a lack of available farms with large numbers of young horses, it was decided that the study would take place in two rounds, on successive foal crops over the course of 2 years.

3.2.4 Enrollment of horses:

A press release was issued to Michigan equine-related publications to recruit large breeding farms into the study (Appendix _). Although participation in the study would provide free deworming products and blood test results to participants, the response to the solicitation was lower than expected. Three equine breeding farms were enrolled in the study. They were the Michigan State University Horse Teaching and Research Center (MSU HTRC) in East Lansing, Michigan, which bred Arabian horses; the MSU Merillat Equine Center in Adrian, Michigan, which bred American Quarter Horses; and a smaller private Arabian farm in Davison, Michigan. In fall, weanling horses were screened for

enrollment using the western blot antibody to *S. neurona* test on serum collected by jugular venipuncture (Rossano et al., 2000). The criterion for a positive test was simultaneous reactivity to the 30- and 16-kDa *S. neurona* antigens. Horses that had only produced antibodies to the 30-kDa antigen were classified as "suspects" who might be in the process of seroconverting. Horses that had no reactivity to either antigen were classified as negative. Horses that tested positive were excluded from participation in the study; horses that tested suspect or negative were enrolled in the study.

Managers of participating farms filled out questionnaires detailing the feeding, housing and preventive health management of each horse in the study. Initial questionnaires (Appendix VIII) were administered at the start of the study and follow-up questionnaires (Appendix IX) were administered at every blood collection date thereafter.

2.2.5 Allocation into treatment groups:

For each farm, horses in the negative and suspect test groups were given ID numbers and a computer was used to generate random numbers to allocate them into treatment groups. In order to minimize between-group variation, negative testers and suspect testers were randomized separately, so that an equal number from each group would be divided between treatments. Horses in group A would received the active drug, pyrantel tartrate in the form of Strongid C 2x®, and horses in group B received a look-alike placebo pellet. To insure that horses receiving the placebo would not suffer ill effects from parasites, all horses in the study received ivermectin at the start of the study and every 2 months after.

3.2.6 Analysis of data:

For purposes of analysis, only horses that tested fully positive on the western blot test were classified as positives. Horses testing suspect or negative were classified as negatives. Results for the 3 herds were combined in a 2 x 2 table and the independence of results by treatment group was tested by the Fisher's exact test (2-tailed). A p-value of ≤ 0.05 was deemed to be significant.

If there had been a sufficient number of observations for a multivariable logistic regression model, one would have been developed to evaluate potential risk factors identified in the horse management data, and the analysis would have been conducted at the individual animal level. Because horses in the study were in herds, and therefore would not represent independent observations, the model would have included a random effects term grouped by herd.

3.2.7 Study timeline:

Weanling horses were screened for enrollment from October to December of 2001. The study protocol required starting the horses on their treatments 2 weeks prior to being retested to assure their serological status upon starting the study. This was done to avoid misclassification error due to seroconversions that occurred due to infections with *S. neurona* that preceded the treatments. Upon testing negative or remaining suspect at the final screening, horses were retested on a bi-monthly basis until they tested positive. Because of a lack of pasture space and staff, the two largest farms, (the MSU HTRC and MSU Merillat) were not able to start feeding treatments until February and March, 2002,

respectively. In order to keep the farms on approximately the same timeline, the small private Arabian farm started the study in February.

Weights were estimated using a weight tape, and doses were determined according to label instructions for Strongid C 2x®. While enrolled in the study, the horses received their treatments daily. On the two largest farms the horses were group-housed by treatment group and fed their treatments mixed in the grain according to label instructions for Strongid C 2x®. There, the doses were based on mean weights per group and the grain with pellets were provided in group feeders. On the smaller farm, horses were group housed during the day, but individually fed their treatments in stalls in the evening. The study was not continued for a second year, and the final blood collection took place in August 2002.

3.3 RESULTS:

3.3.1 Enrollment of horses:

Thirty-four horses on the 3 farms were eligible for the study after testing negative or suspect for antibodies to *S. neurona* on the screening test. Unfortunately, seroconversions that occurred during the period between screening and sampling 2 weeks after starting the treatments resulted in the loss of many horses. This was an unexpected complication for the time of the year (late winter), when opossums are less active. All of the horses (8/8) in Group A and 3 of 7 horses in Group B at the MSU HTRC tested positive for antibodies to *S. neurona* after 2 weeks of treatment. Because there were no horses in Group A to continue the study, the study was stopped there in April, 2002. The first blood collection following 2 weeks of treatment at MSU Merillat showed that 5 of 7

horses in Group A had seroconverted, and 3 of 8 horses in Group B had seroconverted, reducing the sample size at MSU Merillat to 7 horses, one of which was subsequently lost to the study when it was sold. At smaller Arabian farm, 2 of the 6 horses on the trial refused to eat their treatment (1 was in Group A, 1 was in Group B), however the remaining 4 horses did test negative for enrollment in the study. Thus, a total of only 10 horses were available for follow-up.

3.3.2 Statistical analysis:

By August, 2002, only 3 of the 10 horses enrolled had remained seronegative for the duration of the study, 1 in the pyrantel tartrate group and 2 in the placebo group (Table 3.1). No significant difference was found between groups ($P \leq 1.0$). The 4 horses at the small Arabian were all tested on the final day of the study (as opposed to only the 1 remaining negative horse) and over the course of the final 2 months, 2 of 3 seropositive horses (1 from each treatment group) had reverted to seronegative status. Because those horses had already been formally dropped from the study, the changes in status were not included in the final summary table.

Sample size was not sufficient for a multivariable logistic model, so this analysis was not performed.

3.4 DISCUSSION:

The results of this study did not support they hypothesis that daily administration of pyrantel tartrate prevents infection of horses with *S. neurona*, and are inconclusive. The sample size of 10 horses did not provide adequate statistical power to detect a

difference between treatment groups, and this fact should be taken into consideration when interpreting the results.

The lack of ability to obtain timely cooperation from the farm managers when separating horses into treatment groups and scheduling blood collections was a major impediment to the success of the study. This was largely due to a shortage of personnel with time to assist the investigators with catching and identifying horses in pastures. Better results could probably have been obtained if the majority of the horses in the study had been stalled at some point during the day, rather than pastured continuously. That would have allowed for easier identification of the horses by investigators, who could have proceeded to collect blood without assistance from the farm employees. This is not a typical way of housing growing horses on large breeding farms in Michigan, however, so finding herds to fit that study criteria would most likely have been difficult.

It was interesting to see that 2 horses reverted to seronegative status after testing positive 2 months before. If time and funding had allowed, follow-up testing of other seropositive horses in the study might have revealed a pattern of waxing and waning of antibody titers that could have reflected either successful elimination of the parasite followed by re-infection, or the suppression and re-emergence of the parasite due to the existence of a latent or dormant stage in the horse.

The difficulties experienced in this study demonstrate some of the challenges that can be encountered when conducting a controlled field intervention involving horse farms. Future investigators should be aware of how busy horse farm managers are, and try to plan studies that require minimal involvement from them, or provide sufficient compensation to provide a motivation for better compliance with the study protocol.

Table 3.1. Summary of results of antibody tests for horses by the end of the study. Totals for positive horses are for blood samples drawn on the day they were dropped from the study, totals for negative horses are for the last samples drawn.

Treatment Group	**Positive Test**	**Negative Test**	Totals
A (Strongid C®)	3	1	4
B (placebo)	4	2	6
Totals	7	3	10

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CHAPTER 4

PARASITEMIA IN AN IMMUNOCOMPETENT HORSE EXPERIMENTALLY CHALLENGED WITH *SARCOCYSTIS NEURONA* SPOROCYSTS

4.0 ABSTRACT:

Equine protozoal myeloencephalitis (EPM) is a serious neurological disease of horses in the Americas. Most cases are attributed to infection of the central nervous system with *Sarcocystis neurona*. Parasitemia has been documented in one immunocompromised foal, but has not been demonstrated in immunocompetent horses. The objective of this study was to isolate *S. neurona* from the blood of immunocompetent horses.

Horses used in this study had received *S. neurona* sporocysts administered at the following doses and time periods: 100/day for 28 days, followed by 500/day for 28 days, followed by 1,000/day for 42 days. Six yearling colts were selected for attempted culture from blood, 2 testing positive, 2 testing suspect and 2 testing negative for antibodies to *S. neurona,* 2 weeks prior to participating in this study. Two 10 ml tubes with EDTA additive were filled by jugular venipuncture from each horse and allowed to settle at room temperature for approximately 2 hours. For each horse, 3-3 ½ mls of plasma was pipetted from the tops of the tubes and combined in a 15 ml conical centrifuge tube. The centrifuge tubes were subsequently spun for 10 minutes at 2,500 RPM and the plasma was decanted from the tubes and discarded. The pellet was resuspended in plain DMEM

and spun again for 10 minutes at 2,500 RPM. The wash solution was removed and the pellet was resuspended in a medium of 10 ml DMEM, plus 10% fetal bovine serum, HEPES, PenStrepFungizone, L-glutamine and sodium pyruvate. The tubes were stored at room temperature while they were being transported to our laboratory (approximately three days) and were subsequently pipetted onto confluent equine dermal cell cultures and incubated at 37°C with 5% CO_2. The medium was removed the next day and replaced with DMEM with 6% fetal bovine serum with HEPES, PenStrepFungizone, L-glutamine and sodium pyruvate. The cultures were monitored weekly for parasite growth for 12 weeks. Any merozoites grown from cultures were harvested and tested by PCR with RFLP to confirm species identity, and PCR products were sequenced for comparison to known strains of *S. neurona*.

At the conclusion of the study, a culture was isolated from one horse, which was confirmed to be S. neurona. Gene sequence analysis showed that the isolate was identical to the strain with which the horse inoculated, and differed from the known strains. To our knowledge this is the first report of parasitemia with *S. neurona* in an immunocompetent horse.

4.1 INTRODUCTION:

Equine protozoal myeloencephalitis (EPM) is a serious neurological disease of horses in the Americas. Most cases are attributed to infection of the CNS with *Sarcocystis neurona*, although several cases have been due to infection with *Neospora* sp (Dubey et al., 2001a). The definitive host of *S. neurona* in the North America is the Virginia opossum (*Didelphis virginiana*), and intermediate hosts of *S. neurona* identified to date include the domestic cat (Dubey et al., 2000), the nine-banded armadillo (Cheadle et al., 2001a), the striped skunk (Cheadle et al., 2001b), the raccoon (Dubey et al., 2001c) and the sea otter (Enhydra lutris) (Dubey et al., 2001b). Horses are believed to be aberrant hosts to *S. neurona*, and despite the fact that a large proportion (50-60%)of horses that live in areas where there are opossums are seropositive for antibodies to *S. neurona* (Dubey et al., 2001a), the incidence of EPM appears to be low (0.15% nationally) (NAHMS, 2001).

A number of researchers have attempted to experimentally infect horses with *S. neurona* sporocysts, but none have succeeded in recovering the parasite from tissues examined at necropsy (Fenger et al., 1997; Cutler et al., 2001; Saville et al., 2001; Sofaly et al., 2002). One exception has been the demonstration of parasitemia in an Arabian foal with severe combined immunodeficiency (SCID) that was fed 500,000 sporocysts in a single dose (Long et al., 2002). SCID foals lack the ability to mount specific B and T cell immune responses because of a genetic mutation (Shin et al., 1997). *Sarcocystis neurona* was cultured from blood drawn from the foal 21 days post-inoculation (Long et al., 2002).

We hypothesized that *S. neurona* could be cultured from the blood of immunocompetent horses. The purpose of this study was demonstrate parasitemia in immunocompetent horses receiving low daily doses of *S. neurona* sporocysts.

4.2 MATERIALS AND METHODS:

Horses used in the present study were part of a larger study that tested the ability of daily administration of pyrantel tartrate to prevent infection with *S. neurona*. The details of the study has been described elsewhere (Chapter 3 of this dissertation). Briefly, the horses were given oral doses of *S. neurona* sporocysts (strain SN37-R, kindly provided by J.P. Dubey) every day, and this dose was immediately followed by feeding either pyrantel tartrate pellets or a placebo pellet. The treatment was not found to have an effect on seroconversion by treatment group, indicating that it did not kill *S. neurona* at the dose given. Sporocysts were administered at the following doses and time periods: 100/day for 28 days, followed by 500/day for 28 days, followed by 1,000/day for 56 days. Serum was tested weekly and CSF was tested monthly for antibodies to *S. neurona* by western blot at Michigan State University (Rossano et al., 2000). Seroconversions were first seen in 2 colts on day 77 of the study, 14 days after starting the 1,000/sporocysts per day dose. When the present study was conducted, the horses had been receiving 1,000 sporocysts/day for 42 days, and had received their most recent dose of sporocysts approximately 18 hours before blood was collected.

Six yearling colts (ID numbers 7, 33, 42, 64, 105 and 163) were selected for attempted culture from blood, and the selection was made based on western blot test results for serum drawn 2 weeks previously. (Test results from 1 week prior to the study

were not yet available.) Since little is known about the staging of *S. neurona* infection in horses, the goal of the sample selection was to choose horses at different stages of seroconversion in order to increase the chances of detecting parasitemia. Two of the horses had tested negative for serum antibodies to *S. neurona*, 2 horses were in the process of seroconversion and had only produced antibodies to the 30-kDa antigen, and 2 horses had tested positive (producing antibodies to both the 30- and 16-kDa antigens) for serum antibodies to *S. neurona*. The seropositive horses had seroconverted approximately 4 weeks (#163) and 2 weeks (#64) prior to the day culture was attempted. Western blot test results were later obtained for serum and CSF samples taken at the time of culture.

Two 10 ml tubes with EDTA additive were filled by jugular venipuncture from each horse and allowed to settle at room temperature for approximately 2 hours. Three to 3 ½ mls of plasma was gently pipetted from the tops of the tubes and transferred to a 15 ml conical centrifuge tube. This procedure was repeated for the samples from each horse. The centrifuge tubes were then spun for 10 minutes at 2,500 RPM and the clear plasma was decanted from the tubes and discarded. The pellet was resuspended in plain DMEM and spun again for 10 minutes at 2,500 RPM. The wash solution was removed and the pellet was resuspended in a medium of 10 ml DMEM, plus 10% fetal bovine serum, HEPES, PenStrepFungizone, L-glutamine and sodium pyruvate. The tubes were stored at room temperature while they were being transported to MSU (approximately three days) and were subsequently pipetted onto confluent equine dermal cell cultures and incubated at 37°C with 5% CO_2. The medium was removed the next day and replaced with DMEM with 6% fetal bovine serum with HEPES, PenStrepFungizone, L-glutamine and sodium pyruvate. The cultures were monitored weekly for parasite growth for 12 weeks.

Merozoites grown from the cultures were harvested on a weekly basis. First the cell culture medium, including merozoites was pipetted off the culture flask and the medium was spun for 30 minutes at 3,000 RPM. The medium was subsequently pipetted from the tubes and the pellet was resuspended in sterile phosphate buffered saline (PBS) solution. The tubes were spun again for 30 minutes at 3,000 RPM and the PBS was decanted from the pellet. The pellets were then resuspended in 1.5 ml of sterile PBS and transferred to microfuge tubes and spun for 2 minutes at 14,000 RPM. The PBS was decanted from the pellets, and the process was repeated. The PBS was removed from the pellets and the tubes were stored at –80°C for future use.

DNA was extracted from merozoites harvested from cell culture using the QIAGEN Dneasy kit for tissue samples. PCR was performed using primers designed for a *Sarcocystis* species 334 bp product, JN25/JD396, using an annealing temperature of 55°C (Tanhauser et al., 1999). Samples from culture were tested using MIH1 as a positive control and a no-DNA water control. PCR products were cut using restriction enzymes (Tanhauser et al., 1999) to confirm the identity of *S. neurona*.

PCR products confirmed to be *S. neurona* were purified using the QIAquick PCR purification kit and were submitted to the MSU Genomics Technology Support Facility for sequencing. PCR products from a single amplification reaction using DNA isolated from the cultured *S. neurona* isolate were sequenced in both directions. This was performed twice. The Pretty routine of the Wisconsin CGC SeqWeb program was used to produce consensus sequences for each PCR product. The culture sequence was then compared with known strain UCD1 (Genebank #AF093158), MIH1 (Mansfield et al., 2001), and SN37-R. The SeqWeb PileUp routine was used to align the consensus

sequences with sequences from GenBank and MSU. The sequences used for comparison spanned from 201 bp to 450 bp of the PCR target from JNB25/JD396.

4.3 RESULTS:

The culture from horse #64 developed a small plaque in the cell monolayer that was observed to have motile merozoites in it 38 days after being placed on equine dermal cells. The culture continued to grow well and develop multiple plaques. No other flask produced a positive result, and they were discarded after 12 weeks of observation.

When tested by PCR, the #64 isolate produced a 334 bp band that was cut by Hinf I and not by Hind III (Figure 1). This was consistent with results for the MIH1 positive control, a known strain of *S. neurona*. When the PCR products were sequenced, they showed 100% agreement between SN37-R and #64. The SN37-R and #64 isolates differed from UCD1 at one position and from MIH1 at 3 positions (Figure 2).

Western blot antibody tests for serum samples drawn the same day as culture was attempted revealed that when the blood was drawn, 5 horses were seropositive and 1 horse was classified as suspect (Table 1).

4.4 DISCUSSION:

The results obtained in this study suggest that the parasite cultured from horse #64 was the same strain as SN37-R, the strain used to inoculate horses in the study. The PCR with RFLP supports that the isolate is *S. neurona*, and the sequence analysis revealed that while SN37-R and #64 were identical in the region examined, they were distinct from UCD1 and MIH1. To our knowledge, these results represent the second report of

parasitemia in a horse experimentally infected with *S. neurona* (Long et al., 2002) and the first report describing parasitemia in an immunocompetent horse.

The serum antibody test results for the colt demonstrate that viable *S. neurona* can be present in the bloodstream 4 weeks after a horse has mounted a detectable IgG response. Because a daily dosing scheme was employed in the study, it is not possible to determine the time to parasitemia from a single dose of sporocysts. Further studies should pursue this important question. The present study does, however, demonstrate that chronic low doses of sporocysts can be used to produce parasitemia under controlled conditions, and this technique may be useful for future studies of horses' immune responses to *S. neurona*.

Table 4.1. Summary of test results for horses in the study. Treatment groups are (PT) = pyrantel tartrate and (P) = placebo.

Horse ID# and treatment group	Serum antibody test result 14 days prior to culture	Culture result	Serum antibody test result for day of culture	CSF antibody test result for day of culture
7 (P)	Negative	Negative	Positive	Negative
33 (PT)	Negative	Negative	Suspect	Negative
42 (PT)	Suspect	Negative	Positive	Suspect
64 (PT)	Positive	Positive	Positive	Suspect
105 (P)	Suspect	Negative	Positive	Suspect
163 (P)	Positive	Negative	Positive	Positive

Figure 1. Picture of ethidium bromide-stained 1.8% agarose gel showing *Sarcocystis neurona*-specific PCR products and RFLP analysis of MIH1 and #64. Lane 1contains a 100 bp DNA ladder, lane 2 contains MIH1, lane 3 contains MIH1 digested with Hinf I, lane 4 contains MIH1 digested with Hind III, lane 5 contains #64, lane 6 contains #64 digested with Hinf I, lane 7 contains #64 digested with Hind III, and lane 8 contains the negative control.

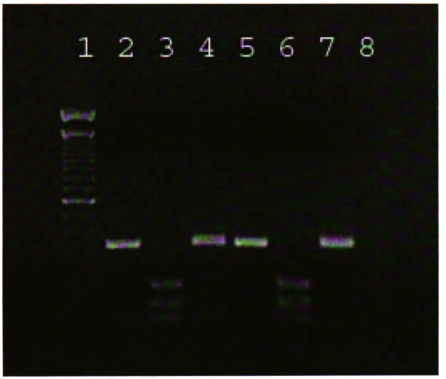
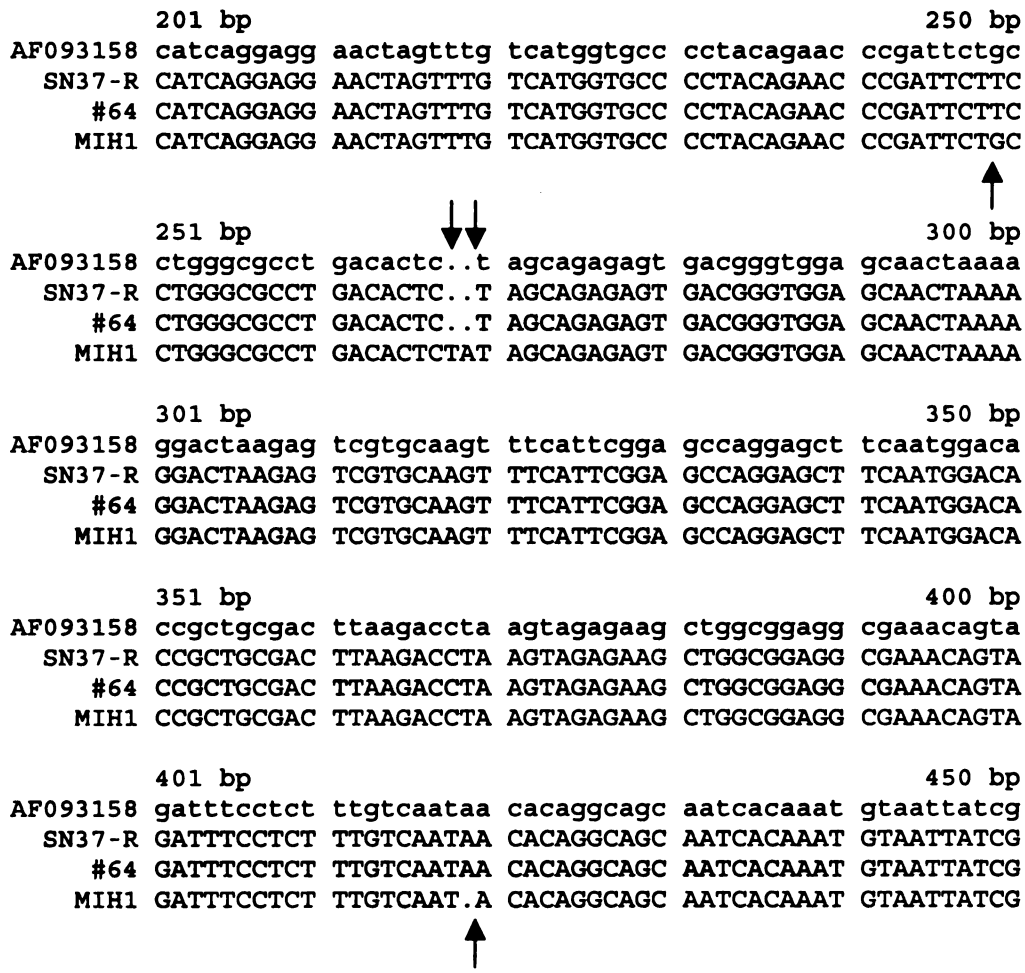


Figure 2. Sequence alignments for data spanning from 201 bp to 450 bp of the PCR target from JNB25/JD396. Row 1 contains the sequence for UCD1 (GenBank # AF093158), row 2 contains the sequence for SN37-R (the isolate administered to experimentally infected horses), row 3 contains the sequence from S. neurona cultured from horse #64 and row 4 contains the sequence of MIH1. The sequences for SN37-R and #64 were identical, but differed from UCD1 and MIH1 at the 249 bp position. The rest of the sequences for SN37-R, #64 and UCD1 were identical, and differed from MIH1 at the 267-268 positions and the 419 position.



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CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 SUMMARY:

These studies have investigated the ability of daily administration of pyrantel tartrate to prevent infection with *S. neurona* in horses by two methods: by an experimental challenge with sporocysts under controlled conditions and by a field intervention to test the performance of the drug against natural infections in an on-farm setting. Neither study supports the working hypothesis that pyrantel tartrate could be used as a preventive agent for EPM.

The final study has demonstrated that *S. neurona* can be cultured from the blood of an immunocompetent horse. If it proves repeatable, the technique described here may have utility in future studies of the immune response of horses to *S. neurona* infection.

5.2 CONCLUSIONS:

The studies described here have addressed the hypotheses stated in Chapter 1:

H_1: Daily pyrantel tartrate administration prevents infection with *S. neurona* in horses.

H_2: Daily pyrantel tartrate administration delays infection with *S. neurona* in horses.

H_3: Daily pyrantel tartrate administration prevents infection with *S. neurona* in horses under field conditions.

H_4: *S. neurona* can be detected in the blood of immunocompetent horses

The results of the experimental challenge study showed that the infection of horses with *S. neurona* was neither prevented nor delayed by daily administration of pyrantel tartrate, thus refuting H_1 and H_2. The results of the field intervention study were less conclusive, due to the small sample size and difficulties in conducting the study, but they do no lend support to H_3. As a whole, the studies suggest that if pyrantel tartrate is to demonstrate efficacy in preventing infection with *S. neurona* in horses, it will most likely not be at the label dose. It is possible that future studies could demonstrate a dose-response if higher doses of pyrantel tartrate are given to horses, or if the drug is given more frequently than once per day.

The results of the experimental challenge study suggest that horses may be less susceptible to *S. neurona* infection than previously thought. This may be because the experimental horses were not appreciably stressed and they had free-choice access to forage, providing for constant gut fill and movement of ingesta. This leads to the questions as to whether natural infections are the result of large doses of sporocysts or an immunosuppressive event that makes some horses more vulnerable to infection, or the practice of feeding horses at intervals rather than free choice.

The field intervention trial illustrates the potential pitfalls of working with horses not owned or controlled by the investigators. There, it was learned that if staff is unavailable to cooperate with the study schedule, the chances of following the protocol are greatly diminished.

The results of the study in which *S. neurona* was cultured from the blood of an immunocompetent horse supports H_4, and has demonstrated that horses with detectable IgG antibodies to the parasite may still not be able to control the infection. This may

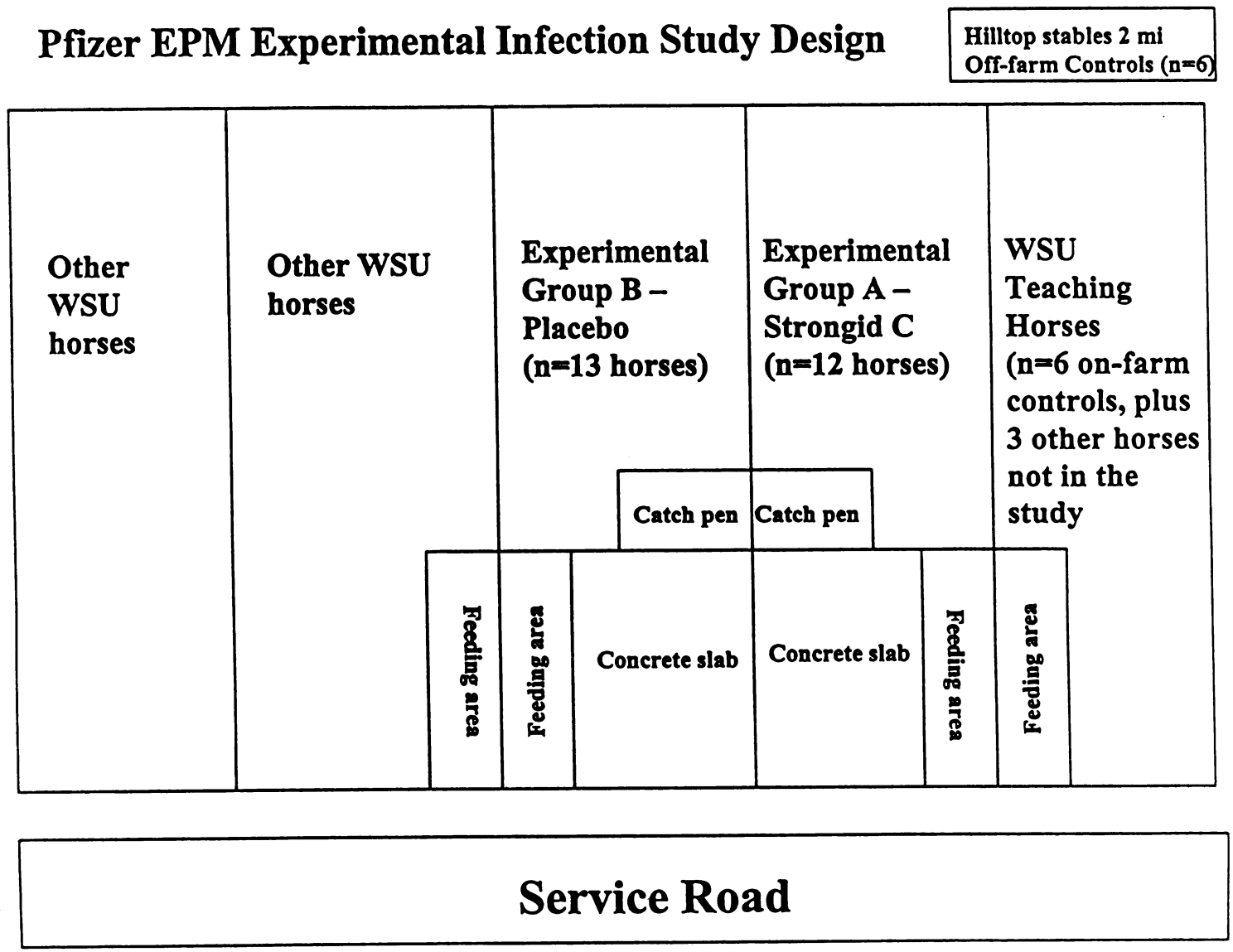
have implications for the use of the EPM vaccine, which stimulates production of IgG antibodies, but has not yet been shown to prevent infection with *S. neurona*.

No new horse management recommendations can be drawn from these studies, other than ruling out daily pyrantel tartrate as an EPM preventative at the label dose for Strongid C 2x, although some horse owners may derive comfort from knowing that horses appear to be naturally resistant to the parasite.

APPENDICES

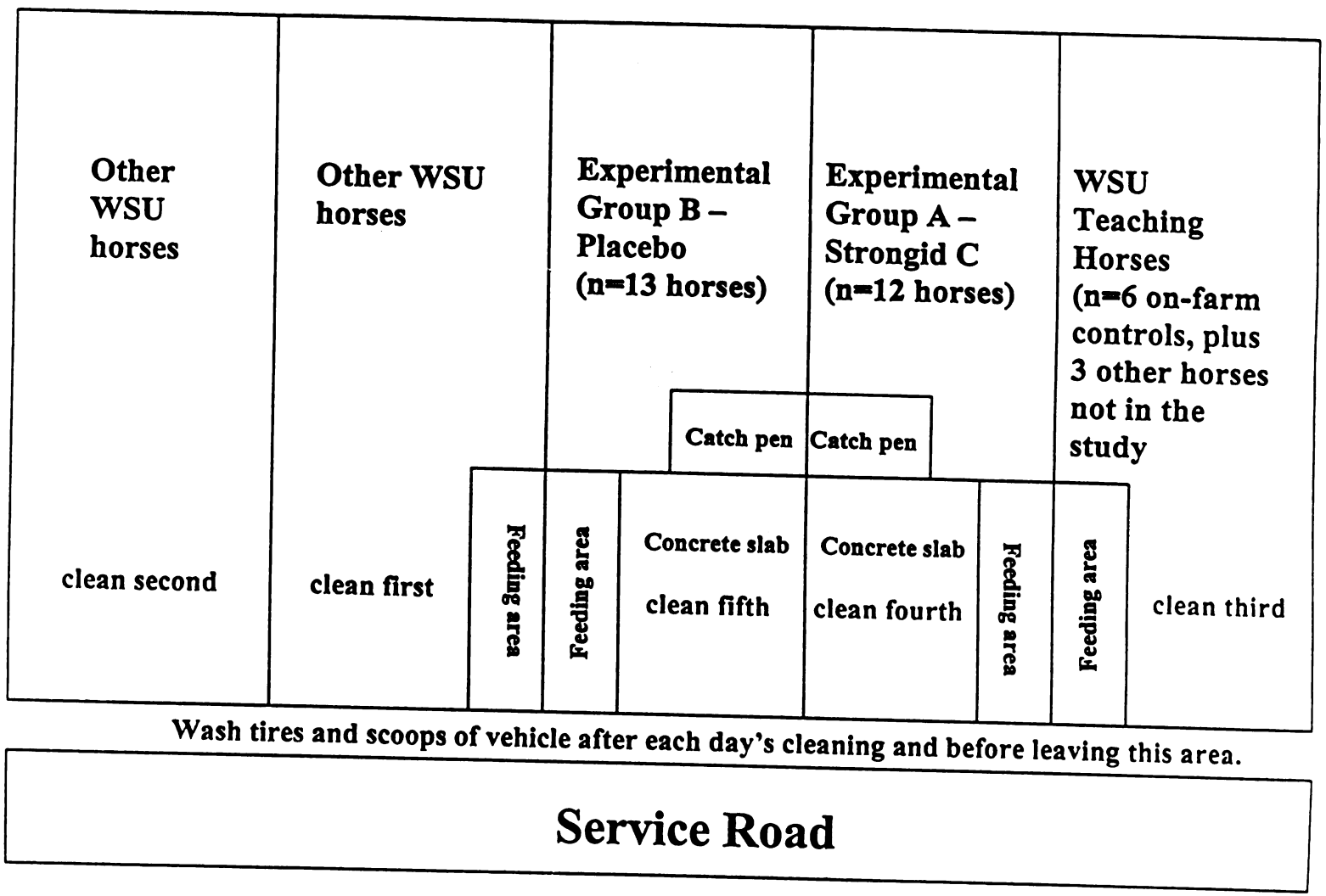
APPENDIX I

Pfizer EPM Experimental Infection Study Design



APPENDIX II

Protocol for Cleaning Pens for EPM/Pfizer Study



APPENDIX III

SPOROCYST DOSING PROTOCOL FOR INFECTION STUDY

Materials needed:
Data sheet for recording treatments
Pipeting ball (rubber)
10 ml pipets
Sporocysts in solution, 3,000 sporocysts/ml concentration
1-liter glass beaker
Corn syrup
Stir plate
Stirring bar
5cc oral syringes
4 oz specimen cups
Latex gloves
Rubber boots
Coveralls

Stock solution:

Gradually add 700 ml corn syrup to 300 ml distilled water in a glass beaker, while stirring at speed #7 on the stir plate. Mix with stir bar until thoroughly blended. Makes 1 liter of 70% corn syrup solution. Store in refrigerator.

Prepare sporocyst inoculum for daily dosing immediately prior to dosing

Procedure:
Wear latex gloves
Add 19 ml of 70% corn syrup solution in specimen cup
Place stir bar in specimen cup and set stir plate to speed #4
Vortex sporocysts in solution at high speed for 30 seconds
Add 1 ml sporocyst solution to specimen cup and allow to stir for 1 minute
Gradually add 40 ml of corn syrup solution (10 ml at a time) to the specimen cup
Stir the sporocyst inoculum for an additional 2 minutes and remove the stir bar
Take the specimen cup to the horse paddocks
Draw 2 ml of sporocyst/syrup mixture into a syringe
Approach a horse that is being held for you
Place the syringe in the corner of the mouth, and quickly squirt the contents to the back of the tongue
Observe the horse to insure that the dose is not spit out
Follow with dose of Strongid treatment (A or B)
When exiting the horse pen, wash your shoes in the footbath
When finished dosing all horses, remove gloves and wash hands
Record treatments on horses' individual record sheets

APPENDIX IV

Neurological Exam Form
Pfizer/EPM Study

Date: _____ Horse Number: _____

Examiner: _____

Behavior: _____

Mentation: _____

Cranial Nerves:

Olfactory (I)	Normal	Abnormal	L	R	Facial/Motor (VII)				
Vision (II)	Normal	Abnormal	L	R	Palpebral	Normal	Abnormal	L	R
Pupil size	Normal	Abnormal	L	R	Ear	Normal	Abnormal	L	R
Pupil light reflex (III)	Normal	Abnormal	L	R	Lip	Normal	Abnormal	L	R
Strabismus (III, IV, VI)	Normal	Abnormal	L	R	Head Tilt (VII)	Normal	Abnormal	L	R
Muscle, Jaw tone (V)	Normal	Abnormal	L	R	Hearing	Normal	Abnormal	L	R
Trigeminal/Sensory (V):					Nystagmus	Normal	Horizontal	Positional	
Opthalmic	Normal	Abnormal	L	R	Swallow/Voice	Normal	Abnormal	L	R
Maxillary	Normal	Abnormal	L	R	Slap Test	Normal	Abnormal	L	R
					Tongue (XII)	Normal	Abnormal	L	R

Symmetry of neck/body: Normal Asymmetric (specify): _____

Gait and Posture: (0 = Normal, 4 = Recumbent)

	Paresis	Ataxia	Hypometria	Hypermetria
LF				
RF				
LH				
RH				

Description of gait: _____

Circling: Left Normal Abnormal

Right Normal Abnormal

Backing: Normal Abnormal

Describe circling and/or backing: _____

Cutaneous sensation: Normal Abnormal

Tail pull: Left Normal Weak

Right Normal Weak

Anal sphincter reflex: Normal Abnormal

Incline: Normal Abnormal

Sweating: Normal Abnormal

Urination: Normal Abnormal

Tail tone: Normal Abnormal

Case assessment: _____

APPENDIX V

Protocol for CSF Collection

Spinal tap procedure:

Preparation of horses:

Horses will be weighed on a livestock scale. Horses will then be clipped along the jugular groove of the neck on both sides. The left side of the neck will be scrubbed with Betadine solution and alcohol.

Placement of catheter:

The catheter site will be injected with 0.5 ml of 2% Lidocaine and allowed to stand for several minutes. The catheter is then inserted in the jugular vein in a routine manner, and an extension set is placed on the catheter.

Anesthesia:

Horses will be randomized into two groups receiving different anesthesia protocols. The study uses a crossover design, and so the horses will receive the alternate protocol for their second spinal tap. Doses of drugs used will be calculated according to the horse's weight.

All horses will be pre-medicated with Xylazine. One group will receive telazol, ketamine and detomadine. The other group will receive ketamine and midazolam. When the drugs take effect, the horse is eased down to the floor of the stall and placed in a position to facilitate the collection of spinal fluid. Horses will be monitored by the antesthesiologist, or a student under his supervision, during the spinal tap procedure and during recovery. Time to recovery data will be collected by those personnel.

Blood Collection:

Once the horse is anesthetized blood can be collected from the catheter. The first 20 cc's of blood are drawn and discarded. A new syringe is placed on the catheter and approximately 90 ccs of blood are collected. 2 EDTA tubes, 2 yellow-topped tubes, 2 red-topped tubes and 3 serum separator tubes decapped, filled and capped shut. Hep saline solution will be flushed through the extension set on the catheter and the stopcock closed.

Spinal Fluid Collection:

The mane and hair coat immediately behind the ears, and over the first two vertebrae of the spine will be clipped and scrubbed with Betadine solution and alcohol. The horse's head is flexed toward the chest. A veterinary clinician will supervise students performing the procedure. Sterile gloves will be put on and a sterile spinal needle will be placed on the midline of the spine at the atlanto-occipital site. The needle will be slowly advanced until the dura mater is punctured and CSF flows from the needle when the stilette is removed. Once the CSF is dripping from the needle, a 13 ml plastic tube will be filled, followed by an EDTA tube and 6 1.8 ml cryo vials. When all the tubes are filled the spinal needle is removed and the procedure is complete.

APPENDIX VI

Necropsy Protocol Pfizer Challenge Study

(L.S. Mansfield 2/17/03)

Purpose: Demonstrate and recover Sarcocystis neurona from infected horses to demonstrate that neurological disease is associated with live parasite with identity to the parasite we used for challenge.

Animals:
Horses with the following numbers from the Pfizer challenge study (27,33 42, 154).

Experimental design:
Groups:

Group #1:	Uninfected	none
Group #2:	S. neurona challenged/Treated	1 horse
Group #3:	S. neurona challenged/Treated	1 horse

Equipment needed:
From Laboratory

- Syringes
  - 60cc syringes - xx
  - 3cc syringes - xx
- Needles
  - 18 gauge – 2 boxes
  - 20 gauge – 2 boxes
- Catheters
  - 18 gauge - 4
  - extension set - 3
- Anesthetics
  - Xylazine – X bottles
  - Fatal Plus – X bottles
- Media
  - Sterile saline
  - DMEM with antibiotics
- Instruments
  - Sterile scalpel blades and handles
  - Sterile forceps
  - Sterile scissors
- Heated transport container
- Fixatives
  - 3.7% Formaldehyde ~200mL
  - dry ice
- Tubes
  - 15cc tubes with labels
  - 50cc tubes with labels
  - cryovials

- Immunohistochemistry
    - Tissue molds
    - OCT compound
- Cooler with ice packs
- Parafilm
- Magic markers
- Culturing supplies
    - Sterile drapes for work surface for handling brain and spinal cord
    - Sterile instruments
    - 50 cc conical plastic tubes

From Barn:
Halters
Twitch
Knives
Saw

Timing:
- Horses will be necropsied on 3/3/03.
- CNS tissue samples will be mailed on 2/x/03 via Federal Express delivery service to:
  Attention Alice Murphy
  A12 Veterinary Medical Center
  College of Veterinary medicine
  Michigan State University
  East Lansing, MI 48824
  517-353-2296
- Alice Murphy will be responsible for culture of CNS tissues when they arrive and will place these on cells on 3/04/03.
- Cells will be examined, brain slurry removed and fresh media placed on cells on 2/x/03.

Necropsy:
1) **Schedule** - On 3/3/03, 3 horses will be euthanatized and necropsied.
2) **Anesthesia**
   a) Horses will be sedated with Xylazine (IM).
   b) Once sedate, the horses will then be euthanized by intravenous overdose of sodium pentobarbital (IV through the catheter). A catheter will be placed in the right jugular vein and Fatal Plus given to effect, ~120 mLs per horse.
3) **Areas to be examined during necropsy**
   a) After death, abdomen will be opened through a midline incision. The diaphragm will be examined and tissues samples recovered.
   b) The **tongue, diaphragm**, and **heart** will be examined and removed for tissue samples.
   c) The **brain** and **spinal cord** will be accessed and samples taken for culture, fixation and snap freezing.
4) **Processing of tissues samples**

a) The **spinal cord** will be placed on a sterile drape on a stainless steel table, and full thickness tissue sections taken from all regions of the cord leaving the dura mater in place as protection to contamination.
b) The **brain** will be accessed through the foramen magnum, the cranium removed and tissue samples taken from cerebrum, cerebellum, and brain stem.
c) **Tools**
   ii) Tools must be sterile for cutting up the brain and spinal cord for culture.
   iii) Tools must not cross contaminate from horses to horse on the other samples
   iv) Tools must not cross contaminate from tissue to tissue in a single horse.
   v) Use you judgment on how to achieve these goals.

5) **Tissue samples will be saved in:**
   a) **Fresh samples to be sent to MSU on wet ice (Make sure to take the samples of cord and brain that flank the formalin sample sites and that center on the area predicted to be damaged based on the clinical examination. Use 50 cc conical tubes for this.)**
      ii) Tongue
      iii) Heart
      iv) Diaphragm
      v) Spinal cord
      vi) Brain (sections of cerebrum, cerebellum, brain stem)
   b) **10% formaldehyde solution (several sections of each for formalin with size roughly 2 cm cubes)**
      ii) Tongue
      iii) Heart
      iv) Diaphragm
      v) Muscles of mastication/chewing (jaw/cheek muscles)
      vi) Muscles of the upper thigh (Semimembranosus, semitendinosus, muscles down the back of the back leg)
      vii) Spinal Cord
      viii) Brain
      ix) Lung
      x) Liver
      xi) Small intestine
   c) **Frozen slowly in O.C.T. compound at -20° for cryosectioning and Immunohistochemistry (Take samples close to the lesion. These should be very small thin sections (1 cm x 0.5 cm) placed in a small plastic bed and covered with OCT compound, then freeze by putting these samples on a tray in a –20 C freezer and letting them freeze until the next day. Then they can be bundled up, put in boxes, and mail on dry ice. These must stay frozen in transit.)**
      ii) Spinal Cord
      iii) Brain
   d) **Snap frozen for later cytokine analysis for RT PCR (Take small samples and freeze in liquid nitrogen or dry ice ASAP)**

ii) Tongue
iii) Heart
iv) Diaphragm
v) Spinal Cord
vi) Brain

Follow-up Procedures Planned:

1) **Culture and PCR to detect *S. neurona***
   a) A tissue sample will be taken from the spinal cord or brain after the horse is euthanized
   b) Half will go into Preston transport media and half into 50 CC tubes for culture
   c) Samples will be ground using a Dounce homogenizer and plated onto ED cells by standard technique (Mansfield et al., 2000).
   d) Merozoites resulting from culture will be tested by a variety of S. neurona specific PCR tests
      ii) ITS Ribosomal targets (Marsh et al., 1998)
      iii) Tanhauser targets and RFLP assays (Tanhauser et al, 1999)

2) **Histopathology**
   a) Formalin (10%) fixed tissues will be paraffin embedded, sectioned, and stained with hematoxylin and eosin.

3) **Tissues collected and snap frozen for PCR**
   a) Samples will be snap frozen, ground in mortar and pestle, and RNA and DNA recovered using a Qiagen Easy recovery kit.
   b) Samples will be tested by PCR as described above.

4) **Tissues collected and frozen at –20C for Immunohistochemistry**
   a) Samples will be taken as small snips, placed in small plastic IHC boats, covered with OCT compound, and frozen slowly by incubation at –20 C.
   b) Frozen sections will be shipped to MSU and given to Matti Kiupal for *S. neurona* IHC.

Blocks will be stored long term in NFSTC floor #1 freezer.

APPENDIX VII

Press Release

Contact: Dr. John Kaneene
(517) 353-5941
or
Mary Rossano
(517) 355-1745

For Immediate Release

The Michigan State University EPM Research Team Seeks Volunteers for Strongid C Study

EAST LANSING, Mich. -- The EPM research team at Michigan State University is currently enrolling breeding farms in a new study that will examine whether the daily dewormer Strongid C helps prevent infection with *Sarcocystis neurona*, the parasite that causes EPM (equine protozoal myeloencephalitis).

The study will focus on weanlings, and will require individual feeding once per day for approximately one year. Ivermectin will be provided for all enrolled horses every eight weeks. Half of the weanlings will receive Strongid C and half will receive a look-alike placebo.

Serum samples will be collected at the beginning of the study and every two months thereafter for testing for antibodies against *S. neurona*. The deworming treatments, Strongid C and *S. neurona* testing will be provided to study participants at no cost to them.

The researchers are hoping to recruit breeding farms that expect to have at least 12 weanlings this fall.

For more information about participating in this study contact Dr. John Kaneene, Director of the Population Medicine Center at the Michigan State University College of Veterinary Medicine at (517) 353-5941.

####

APPENDIX VIII

INITIAL QUESTIONNAIRE

Equine Protozoal Myeloencephalitis (EPM)/Strongid C Study
Michigan State University College of Veterinary Medicine

Farm ID: _____ Date: ____/____/____

GENERAL INFORMATION:

1.) Horse name/ID: _____ 2.) Breed: _____

3.) Birth date: ____/____/____ 4.) Gender: _____ 5.) Treament group: _____

HEALTH INFORMATION:

6.) Date of last paste or liquid deworming: _____ 7.) Type of dewormer used: _____

8.) What vaccinations has this horse received since January 1, 2001? (Check *all* that apply.)

_____ Tetanus _____ EEE/WEE _____ Rabies _____ Flu _____ Potomac Horse Fever

_____ Rhino _____ Strep (strangles) Others: _____

9.) Has this horse ever been vaccinated for EPM? Yes / No 10.) If yes, date of vaccination: _____

MANAGEMENT INFORMATION:

11.) How many horses are kept at this horse's home/facility? _____

12.) Is this horse housed in a barn? Yes / No 13.) Does this horse have access to pasture? Yes / No

14.) Does this horse have access to a dirt lot or other outdoor exercise area that isn't a pasture? Yes / No

15.) If turned out, how many other horses on average are with this horse? _____<5 _____6-10 _____>10

16.) Please describe the type(s) of hay(s) that is/are fed to this horse and indicate what county they come from:

_____

17.) Please describe the type(s) of grains that is/are fed to this horse (Check *all* that apply):

_____ pelleted grains _____ sweet feed other: _____

18.) How many times is this horse fed per day? _____ Grain _____ Hay

19.) How is water provided to this horse? (Check *all* that apply)

_____ individually/ bucket _____ individually/tank _____ with other horses/ tank

_____individually/ automatic waterer _____ with other horses/ automatic waterer

_____ natural surface water Please describe (creek, pond, etc.): _____

20.) Have you ever seen an opossum (alive or dead) within 1 mile of this farm? (circle one) Yes / No

APPENDIX IX

FOLLOW-UP QUESTIONNAIRE

Equine Protozoal Myeloencephalitis (EPM)/Strongid C Study
Michigan State University College of Veterinary Medicine

Farm ID: _____ Date: ____/____/____

GENERAL INFORMATION:

1.) Horse name/ID: _____ 2.) Treament group: _____

HEALTH INFORMATION UPDATE:

6.) Date of last paste or liquid deworming: _____ 7.) Type of dewormer used: _____

8.) What vaccinations has this horse received since the last blood collection? (Check ***all*** that apply.)

_____ Tetanus _____ EEE/WEE _____ Rabies _____ Flu _____ Potomac Horse Fever

_____ Rhino _____ Strep (strangles) Others:_____

MANAGEMENT INFORMATION UPDATE:

9.) How many horses are kept at this horse's home/facility? _____

10.) Is this horse housed in a barn? Yes / No 11.) Does this horse have access to pasture? Yes / No

12.) Does this horse have access to a dirt lot or other outdoor exercise area that isn't a pasture? Yes / No

13.) If turned out, how many other horses on average are with this horse? _____<5 _____6-10 _____>10

14.) Please describe the type(s) of hay(s) that is/are fed to this horse and indicate what county they come from:

_____

15.) Please describe the type(s) of grains that is/are fed to this horse (Check ***all*** that apply):

_____ pelleted grains _____ sweet feed other: _____

16.) Please describe the type(s) of supplements that is/are fed to this horse:_____

_____

17.) How many times is this horse fed per day? _____ Grain _____ Hay

18.) How is water provided to this horse? (Check ***all*** that apply)

_____ individually/ bucket _____ individually/tank _____ with other horses/ tank

_____individually/ automatic waterer _____ with other horses/ automatic waterer

_____ natural surface water Please describe (creek, pond, etc.): _____