DEVELOPMENT AND EVALUATION OF A SARCOCYSTIS NEURONA-
SPECIFIC IgM CAPTURE ENZYME LINKED IMMUNOSORBENT ASSAY

A Thesis

Presented in Partial Fulfillment of the Requirements for

the Degree Master of Science in the

Graduate School of The Ohio State University

By

Jamie Ellen Murphy, VMD

*****

The Ohio State University
2005

Master’s Examination Committee:
Dr. Stephen M. Reed, Adviser
Dr. William J. A. Saville
Dr. Antoinette E. Marsh

Approved by:
Adviser
Graduate Program in
Department of Veterinary
Clinical Sciences
ABSTRACT

Equine protozoal myeloencephalitis (EPM) is a serious neurologic disease of horses caused primarily by the protozoal parasite *Sarcocystis neurona*. The parasite causes asymmetric neurologic deficits in horses in both North and South America. EPM has a significant economic impact on the US horse industry, with estimated costs from $55.4 to $110.8 million per year.

Currently, antemortem testing is limited in its specificity in exposed and diseased horses. This paper reports on the development of an IgM capture enzyme linked immunoassay (ELISA) for the identification of acute infection with *S. neurona*. The ELISA was based on the *S. neurona* low molecular weight protein SNUCD-1 antigen and the monoclonal antibody 2G5 labeled with horseradish peroxidase. The test was evaluated using serum and CSF from 12 horses experimentally infected with 1.5 million *S. neurona* sporocysts and 16 horses experimentally infected with varying doses (100 to 100,000) of *S. neurona* sporocysts, all of whose histopathology results were available.

The hypothesis of this study was that serum and cerebrospinal fluid (CSF) of horses experimentally challenged with *S. neurona* would have an increased *S. neurona*-specific IgM (Sn-IgM) concentration relative to their pre-infected state. For horses challenged with 1.5 million sporocysts, the results indicate a significant increase in serum Sn-IgM concentrations relative to pre-inoculation for weeks 2 through 6 post-inoculation.
For horses inoculated with lower doses of *S. neurona*, there were significant increases in Sn-IgM concentration at various points in time depending on the inoculation dose. Finally, there was a significant increase between the pre- and post-inoculation CSF Sn-IgM concentrations. Our results support the assay as a valuable addition as a diagnostic tool during the acute phase of EPM.
This thesis is dedicated to Cheyney, Tim, Linda, Brian, Jimmy, and to the memory of Dr. Robert Richardson, III, who was a fantastic friend, matchmaker and veterinarian.
ACKNOWLEDGMENTS

I want to thank my committee for their support as I learned my way around a laboratory. This project was a challenge, which was made enjoyable by their support and intellect.

Thank you to my advisor, Dr. Reed, not only for the support during my thesis, but for sharing his remarkable knowledge of equine internal medicine and his gift of optimism. Neither my masters nor my residency would have been as enjoyable, without his guidance and support.

To Dr. Saville, thank you for the opportunity and challenge of being involved with EPM research.

To Dr. Marsh, thank you for your unending patience in teaching me laboratory procedures. This project would have been impossible without your guidance.

In addition, I would like to thank Drs. Kohn, Toribio and Hinchcliff for the support and mentorship throughout my residency.

Thank you to my husband Cheyney who always takes care of me as I pile more onto my plate. I hope that one day soon, I will be able to make dinner for you.

Finally, thank you to Dr. Reed and Dr. Saville for a lovely honeymoon.
VITA

August 28, 1974 .................................................. Born – Pittsburgh, Pennsylvania

1996 .................................................................................................. B.S., University of Vermont

2001 .................................................................................................. V.M.D., University of Pennsylvania

2001-2002 .................................................................................. Intern, Rood & Riddle Equine Hospital

2002-present ............................................................... Resident, Equine Internal Medicine, The Ohio State University

2002-present ........................................................................... Graduate Student, The Ohio State University

FIELDS OF STUDY

Major Field: Veterinary Medicine
# TABLE OF CONTENTS

Abstract ........................................................................................................ ii  
Dedication ........................................................................................................ iv  
Acknowledgments ............................................................................................. v  
Vita ................................................................................................................. vi  
List of Figures ................................................................................................... ix  

1. Introduction .................................................................................................. 1  
   1.1. Literature Cited ..................................................................................... 2  

2. Literature Review .......................................................................................... 5  
   2.1. History of Equine Protozoal Myeloencephalitis ...................................... 5  
   2.2. Hosts of *Sarcocystis neurona* .............................................................. 7  
       2.2.1. Identifying the definitive host .......................................................... 7  
       2.2.2. Identifying intermediate hosts ......................................................... 8  
       2.2.3. Aberrant Hosts .............................................................................. 9  
   2.3. Other Causative Organisms .................................................................. 9  
   2.4. Life Cycle .............................................................................................. 9  
   2.5. Transmission to the horse .................................................................... 11  
   2.6. Ultrastructure of *S. neurona* ............................................................. 12  
   2.7. Pathology .............................................................................................. 12  
   2.8. Neuroimmunology ................................................................................ 13  
   2.9. Diagnosis .............................................................................................. 16  
       2.9.1. General Information ....................................................................... 16  
       2.9.2. CSF indices ................................................................................... 16  
       2.9.3. Indirect fluorescent antibody titer ................................................... 17  
       2.9.4. Immunoblots ................................................................................ 18  
       2.9.5. Polymerase Chain reaction ............................................................ 19  
       2.9.6. Clinical Signs ............................................................................... 19  
   2.10. Therapy ............................................................................................... 20  
       2.10.1. General Information ................................................................. 20  
       2.10.2. Pyramethamine .............................................................. 21
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Western blot results comparing pre-inoculation samples (Group 1A) (Lanes 1-6), post-inoculation samples (Lanes 1-6), negative control (Lane 7) and positive control (Lane 8).</td>
</tr>
<tr>
<td>3.2</td>
<td>Groups 1A and 1B average PP values from week 0 to week 7. Weeks 0 - 4 had 12 horses (Groups 1A and 1B) at each time point; weeks 5 - 7 had 6 horses (Group 1B) at each time point. (Error bars represent two standard errors.)</td>
</tr>
<tr>
<td>3.3</td>
<td>Groups 2B, 2C, 2D and 2E PP values from week 0 to week 4. (Error bars represent two standard errors.)</td>
</tr>
<tr>
<td>3.4</td>
<td>Box and whisker plots of PP values for pre-inoculation CSF of Group 1A; post-inoculation CSF of Group 1A; pre-inoculation CSF of Group 1B; and post-inoculation CSF of Group 1B</td>
</tr>
<tr>
<td>3.5</td>
<td>Box and whisker plots of OD values for negative and positive control horses and WNV positive horses</td>
</tr>
<tr>
<td>3.6</td>
<td>Correlation between neurologic score and PP. ($R^2$ of 0.2932)</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

Equine Protozoal Myeloencephalitis (EPM) is a serious neurologic disease caused primarily by Sarcocystis neurona [1, 2]. EPM is considered a progressively debilitating disease causing acute to slowly progressive, focal or multifocal neurologic deficits, and is the most commonly diagnosed neurologic disease of the horse [1, 3]. The definitive host of S. neurona is the North American opossum (Didelphis virginiana) [4-6], and the natural intermediate hosts include the raccoon, cat, skunk, armadillo, and sea otter [1, 4, 7-11]. Horses become infected by ingesting sporocysts, which contaminate their environment via opossum feces, and are considered an aberrant host because no sarcocysts are found in their muscle tissue [3].

Currently, the gold standard diagnostic test for EPM is postmortem immunohistochemistry of the gray and white matter. Western blot is an antibody-based test that detects IgG specific for S. neurona in equine serum and CSF, and is one of the only antemortem tests presently available. The test has excellent sensitivity, but its downfall is its specificity for identifying active disease [12, 13]. Clinically, results of serum and CSF Western blot testing are useful for ruling out EPM as a cause of clinical signs; however veterinarians are left with few options for detecting a true positive. With a
reported seroprevalence of infection with *S. neurona* higher than 50% in some regions [14-16], being able to classify animals as exposed or diseased is crucial. Currently, there are insufficient diagnostic tools for identifying infection with *S. neurona*, which likely causes overestimation of diseased horses and inappropriate treatment of horses without active *S. neurona* infections.

In general, serum and CSF IgM testing is reserved for the identification of acutely infected individuals. Activation of humoral immunity by a primary exposure to intracellular parasite antigen initiates a strong IgM response in other Apicomplexan organisms [17-19], and has been applied as a diagnostic tool for several organisms [20-22]. The ability to develop and evaluate a new, more specific diagnostic tool is a crucial step in disease identification. The purpose of the present study was to develop an *S. neurona*-specific IgM capture ELISA and evaluate its usefulness in detecting acute EPM infections in naïve individuals using either serum or CSF.

1.1. Literature Cited


17. Peeters, J.E., et al., Cryptosporidium parvum in calves: kinetics and immunoblot analysis of specific serum and local antibody responses (immunoglobulin A [IgA].


CHAPTER 2

Literature Review

2.1. History of Equine Protozoal Myeloencephalitis

Equine Protozoal Myeloencephalitis (EPM) is a serious neurologic disease of horses caused primarily by the Apicomplexan parasite *Sarcocystis neurona*. The parasite causes asymmetric neurologic deficits in horses in both North and South America. EPM has a significant economic impact on the US horse industry, with estimated costs from $55.4 to $110.8 million per year [1].

The equine neurologic disease was first described in Kentucky by Rooney et al as a segmental myelitis, although it was later renamed “focal encephalitis-myelitis”. A review of 52 cases described asymmetric, progressive spinal ataxia that most frequently affected Standardbreds less than 4 years of age. Initially, no etiologic agent was identified [2].

A protozoal agent was first associated with the characteristic lesions by several separate researchers in 1974, and at that time the protozoa was considered to be *Toxoplasma gondii* [3, 4]; however, evaluation of serum and cerebrospinal fluid (CSF) for antibodies to *T. gondii* were negative [4]. Dubey et al distinguished the protozoan from *T. gondii* in his report, as well as by reexamining Cusick’s original paper [5].
Inequities between the new organism and *T. gondii* included differences in lesion distribution and characteristics, host reaction, and structural and staining differences. Mayhew et al tested sera from horses suspected of the protozoal neurologic disease and discovered that a large percentage of those horses reacted with *Sarcocystis cruzi* antigen using an indirect hemagglutination test, which suggested that the organism may be related to a *Sarcocystis* species [6]. Beech and Dodd named the disease equine protozoal encephalomyelitis, but Mayhew’s coining of equine protozoal myeloencephalitis has maintained popularity.

Attempts to reproduce EPM experimentally initially failed. Oral inoculation using oocysts or sporocysts of several Apicomplexan parasites, including *Sarcocystis* species failed to recreate the disease or determine the causative organism [7]. *Sarcocystis neurona* was eventually identified and named as the etiologic agent of EPM in 1991 when Dubey et al isolated the organism from a horse from New York [8]. The new organism did not react with sera against *T. gondii, Hammondia hammoni, Caryospora bigenetica* or *Neospora caninum*, but did react positively with *S. cruzi* antiserum. The positive reactivity of *S. neurona* and *S. cruzi* supported a close antigenic relationship within the genus *Sarcocystis*. Davis et al also isolated the organism that same year and for the first time the organism’s growth was studied in bovine monocyte cell cultures [9].

Further phylogenetic analysis of *S. neurona* included gene sequencing, which enabled a separation of organisms with similar antigenic identities. The small subunit ribosomal RNA (SSURNA) gene of *S. neurona* was amplified using polymerase chain reaction (PCR) techniques and compared to partial sequences from *Sarcocystis muris, Sarcocystis gigantea, Sarcocystis cruzi, Sarcocystis capracaenis, Sarcocystis tenella,*
Sarcocystis arieticanis, T. gondii, Eimeria tenella, and Cryptosporidium parvum [10].

This study confirmed that S. neurona was from the genus Sarcocystis and elaborated on the similarities between S. neurona and S. muris, S. gigantea and T. gondii species.

2.2. Hosts of Sarcocystis neurona

2.2.1. Identifying the definitive host

In an attempt to identify the definitive host for EPM, a PCR study using feces and gastrointestinal contents from feral raccoons (4), opossums (2), skunks (7), cats (6), hawk (1) and coyote (1) were screened using S. neurona SSURNA [11]. The dramatic homology between the opossum and the S. neurona SSURNA gene suggested that the opossum might harbor the organism. Opossums naturally carry several Sarcocystis spp in their intestinal tract, and unfortunately, S. neurona and S. falcatula were identified as the same organism via gene sequencing by Dame et al in 1995 [12]. A separate study by Fenger et al supported those findings based on an oral inoculation challenge of budgerigars and foals using sporocysts from a feral opossum [13]. Both the birds (18/18) and foals (3/5) developed Sarcocystosis, suggesting that opossum was the definitive host for S. neurona and S. falcatula, and that they were likely the same organism. In hindsight, it is probable that the feral opossums carried both Sarcocystis spp, and the birds and foals were challenged with multiple organisms. Since that time, S. neurona and S. falcatula have been differentiated by DNA sequencing and differing morphology in cell culture [14-16].

Fenger et al solidified the opossum as the definitive host for EPM by experimentally challenging horses with sporocysts obtained from feral opossums and inducing S. neurona seroconversion and neurologic deficits [13]. In addition, the South
American opossum, *Didelphis albiventris*, was identified as another natural definitive host for *S. neurona* in 2001 [17].

2.2.2. Identifying intermediate hosts

Identification of intermediate hosts is still ongoing. Initially, when *S. neurona* and *S. falcataula* were thought to be homologous, researchers assumed that EPM would have avian intermediate hosts. Because of EPM’s geographical distribution, species limited to the western hemisphere were the focus of attention. The life cycle was first completed by Dubey et al using a domestic cat (*Felis domesticus*) as the intermediate host [18], but their role in natural infections was unknown. In a study used to determine if cats were involved in the natural life cycle of EPM, nine feral cats were evaluated for anti-*S. neurona* antibodies. One cat was identified as having antibodies reacting to *S. neurona* antigens, and its muscle tissue containing sarcocysts was fed to an opossum (*Didelphis virginiana*). The opossum shed sporocysts, which were then fed to gamma-interferon receptor knockout (KO) mice, which then developed neurologic disease [19]. Further research by Stanek et al identified a high seroprevalence of anti-*S. neurona* antibodies in feral and domestic cats in Ohio [20]. These findings also support that cats are likely natural intermediate hosts.

Currently, other known intermediate hosts include the nine-banded armadillo (*Dasypus novemcinctus*) [21], the striped skunk (*Mephitis mephitis*) [22], the raccoon (*Procyon lotor*) [23, 24], and the sea otter (*Enhydra lutris*) [25]. The intermediate host range is likely more extensive and further research may elaborate on current knowledge.
Uncommonly, intermediate hosts can develop clinical disease associated with infection with *S. neurona*. Reported neurologic disease has been discovered in cats, a striped skunk, and Southern sea otters [26, 27]

2.2.3. **Aberrant Hosts**

Aberrant hosts are defined as hosts of *S. neurona* that do not produce infective tissue sarcocysts. In addition to the horse, several other species serve as aberrant host for *S. neurona* and develop clinical syndromes not unlike EPM. *S. neurona*-like encephalitis has been seen in a mink, rhesus monkeys, chickens, dogs, sheep, a golden eagle and raccoons [28-32].

2.3. **Other Causative Organisms**

*Neospora* is an important cause of abortion in cattle and is structurally similar to *S. neurona* and *T. gondii*. Several reports also identify *Neospora* spp. as a possible, although uncommon, agent of EPM or a disease similar to EPM [33-35]. The life cycle of *N. caninum* and *N. hughesi* in horses is not well understood; however, the definitive host for *N. caninum* is likely the dog [36]. The typical tissue cysts seen with *Neospora* infections are uncommonly identified in horses with EPM, and it is unlikely that *Neospora* is an important cause of the clinical syndrome [37].

2.4. **Life Cycle**

*Sarcocystis neurona* is protozoan parasite, which belongs to the family Coccidia and subphylum Apicomplexan. *Sarcocystis neurona* is dissimilar from other Sarcocystis species because it can infect a large number of intermediate hosts. The organism causes neurologic disease in horses, as well as EPM-like disease in other aberrant host animals as mentioned above [24, 27-32]. The life cycle was incompletely known until Dubey et
al completed the life cycle using domestic cats (*Felis domesticus*) as experimental intermediate hosts in 2000 [18].

*Sarcocystis* species have an obligatory prey-predator two-host life cycle, with a definitive host and an intermediate host. As with other *Sarcocystis* species, *S. neurona* has a heteroxenous life cycle, with sexual reproduction within the definitive host and asexual reproduction within the intermediate hosts. The definitive host of *S. neurona* is the North American opossum (*Didelphis virginiana*), which ingests the sarcocysts when they prey on the intermediate host. After ingestion of the sarcocyst, the definitive host rarely shows signs of neurologic disease.

The natural intermediate hosts of *S. neurona* include the raccoon, cat, skunk, armadillo, and sea otter [19-25]. Intermediate hosts ingest the sporocysts, most likely from their environment, which leads to sarcocyst formation in their striated muscle. Although atypical, intermediate hosts can have severe disease associated with infection. Once ingested, the sporocyst releases four sporozoites, which likely excyst in the small intestine, migrate through the intestinal wall, gain access to the lymphatic or blood vascular system, invade endothelial cells, and perform two generations of asexual reproduction (schizogony). After asexual reproduction, the organism organizes into sarcocysts within the skeletal muscle. Sarcocyst maturation within the muscle tissue is prolonged. Not all *S. neurona* sarcocysts were mature 144 days post-infection in an experimental model using the domestic cat [18]. Sarcocysts are the only developmental stage of the parasite that is naturally infectious for the definitive host [38].

Horses, and other aberrant hosts, become infected with *S. neurona* by ingesting sporocysts, which contaminate the environment via opossum feces. The pathophysiology
within the horse is currently unknown, although current studies may shed new light on how the organism travels from the gastrointestinal system to the central nervous system [Saville, pers comm.]. To enter into the CNS, the merozoites must penetrate the blood brain barrier (BBB), and it is currently unknown whether they cross within leukocytes or directly through the cytoplasm of endothelial cells. Based on findings in other Sarcocystis species, there are multiple generations of asexual reproduction (schizogony), followed by an eventual invasion of CNS [1, 8]. Only asexual stages have been identified in the aberrant hosts, and they are limited to the CNS. Merozoites can multiply in the CNS of horses for months and invade both neural and inflammatory cells [1].

2.5. Transmission to the horse

S. neurona is known to be transmitted by the fecal-oral route of horses ingesting opossum feces [13, 39-41]. Transmission from the intermediate host or from other horses to the horse is not possible. However, after inoculation with a large dose of sporocysts, sporocysts can pass unexcysted in the horse’s feces and may be potentially infective to control horses [40, 41]. Sporocysts shed by opossums are immediately infective and can contaminate any number of feed or water sources.

Researchers in the 1980’s suggested that birds may spread the sporocyst, as with S. falcatula, but the involvement of an avian species in EPM has never been proven [42, 43]. In addition, insect vectors, such as flies and cockroaches, could be involved in the transmission of S. neurona, as they are for T. gondii [44, 45], but this has not been established.
2.6. Ultrastructure of *S. neurona*

*Sarcocystis neurona* is different from other *Sarcocystis* spp., as well as being distinct from *T. gondii* and *Neospora* spp. In studies to date, schizonts and merozoites, which are the only stages known to occur within the horse, are located within the host cell cytoplasm. They are believed not to have a parasitophorous vacuole at any stage of development, which distinguishes them from *Neospora caninum*. The schizonts contain a prominent residual body and the merozoites are typically arranged in a rosette pattern around that structure [8].

Merozoites multiply by a specialized form of schizogony called endopolygeny, where the nucleus becomes lobulated. The lobes are connected by chromatin strands and can be arranged in groups. As the merozoites develop into schizonts, their nuclei enlarge and divide into four or more nucleoli. Several merozoites develop within the cell and can mature to schizonts within the same host cell. Some merozoites bud from their host cells, penetrate other cells and undergo additional asexual reproduction. Unlike *T. gondii*, merozoites of *S. neurona* lack rhoptries, which was one of the first indications in the 1970's that the organisms were not homologous. The sporocysts (after sporulation) have a thin wall, contain 4 sporozoites, and a residual body [18]. The tissue cysts stain negative with periodic acid Schiff (PAS) stain, as well as negative with Wilder’s silver impregnation, which can differentiate *S. neurona* from *T. gondii*.

2.7. Pathology

Pathologic changes caused by *Sarcocystis neurona* include gross malacia and focal hemorrhage, and are confined to the CNS [3-5]. Most commonly, lesions are seen in the spinal cord, but may also be seen in the brainstem and other areas of the brain [1].
Microscopic changes are often asymmetric, multi-focal to coalescing areas of hemorrhage, non-suppurative inflammatory lesions with lymphoid perivascular cuffing, gliosis and neuronal degeneration [3, 5, 6, 46]. In acute disease, lesions may consist of focal hemorrhage, while in chronic disease there may be areas of varying discoloration [1]. Using routine staining techniques, histopathologic examination may demonstrate merozoites in neutrophils, microglia, macrophages or vascular endothelial cells of the CNS. However, these are found in a small portion of cases. Unlike S. fayeri and other Sarcocystis spp, S. neurona is rarely seen in the vascular endothelium [47, 48]. Uncommonly, S. neurona can be cultured from the central nervous system of a horse with EPM, as shown by Davis et al [9]. Immunohistochemistry can more proficiently identify S. neurona within the CNS [26]. Initially, anti-S. cruzi antibodies were used for immunohistochemistry; however, the development of S. neurona-specific anti-sera has been utilized in more recent studies [49].

2.8. Neuroimmunology

It is well-established that the immune response within the CNS is different from the peripheral tissues, and thus the CNS is considered “immune privileged”. Immune privilege does not imply that the CNS is immunologically inept, but that it is unique and somewhat isolated in its immunoreactivity. It is separated from the systemic blood by the blood-brain barrier, which is a diffusion barrier formed by tight junctions between capillary endothelial cells. The major cell groups in the CNS are neurons and glial cells. Neurons are cells specialized for the conduction and transmission of electrical signals in the nervous system, while glial cells are the support cells of the nervous system and include astrocytes, oligodendrocytes and microglial cells. Microglia are the antigen
presenting cells of the CNS, and are essential in cases of inflammation and infection. Also, the brain endothelial cells themselves can produce and respond to immunoregulatory molecules which may alter the BBB.

During inflammation cells can selectively invade the CNS, but the CNS is also able to mount its own immune response, although it differs from that in peripheral tissue. Studies in laboratory animals indicate that the microenvironment of the CNS supports antigen-directed antibody development in the face of a normal BBB [50]. When antigen is directly placed into the CSF, antigen-specific antibodies appear in the serum and antigen can be recovered in the deep cervical lymph nodes following injection into the CSF [51, 52]. The CNS itself is relatively inaccessible for many diagnostic tests used for other organ systems, making CSF a common indirect measure of changes within the CNS.

There are two branches of the acquired immune system, including humoral immunity and cell-mediated immunity. Humoral immunity is mediated by serum antibodies, which are produced and secreted by B-cells. There are five major classes of immunoglobulins, including IgG, IgM, IgA, IgE, and IgD. IgM is typically the major immunoglobulin produced during a primary exposure to an antigen. It is also produced in a secondary response, but this may be masked by a robust IgG response. Typically, IgM is found in the second highest concentration after IgG in most mammalian serum. Because of its pentameric structure, IgM molecules are efficient agglutinating antibodies. Also, because of its relatively large size (900 kDa), IgM molecules are usually confined to the bloodstream and are likely unimportant in conferring protection in the tissue [53].
Finally, some researchers prefer IgM for antibody testing purpose because of the lessened degree of cross-reactivity as seen with IgG molecules [54].

Although antibody protection likely contributes to the protection of hosts from *S. neurona*, antibody concentration alone does not correlate with protection [55]. Several studies suggest that cell-mediated immunity is crucial for curtailing or preventing EPM, as well as other diseases caused by intracellular pathogens [13, 38, 55]. As discussed by Tornquist et al, the fact that IFN-γ knockout mice develop neurologic disease and histopathologic lesions similar to those in horses infected with *S. neurona* is supportive of the importance of cell-mediated immunity (CMI) in disease control [55, 56]. Also, studies have demonstrated impaired CMI function in horses with clinical EPM suggesting that impaired CMI may exacerbate clinical disease [55, 57]. EPM causes immuno-suppression, as suggested by in vitro impairment of lymphoblast transformation [58], and suppressed γIFN production [57]. Furr et al identified decreased levels of transforming growth factor-β_2 (TGF-β_2) in the CSF of horses with natural infections of EPM [59]. TGF-β_2 likely changes the immune response to infection because it can mediate alterations in adhesion molecules, antigen presenting cells and other cytokines, and has pro-inflammatory properties in low concentrations [59]. The regulation of TGF- β_2 and other cytokines is important for balancing the reaction to protozoans. Changes in the immune response and cytokine production has been reported with other protozoan organism infections of the CNS, including infection with *T. gondii* and *Leishmania* spp. [60, 61]. In addition, γIFN has been shown to play a crucial protective role in other parasitic infections such as *Eimeria tenella* and *Plasmodium chabaudi* [62, 63].
S. neurona infection has also been associated with lower proportion of CD4+ cells than seen in asymptomatic and non-infected horses, although the mechanism has not been determined [55]. Nitric oxide, which is hypothesized to be a part of protection from intracellular parasites, has been shown to be decreased in the CNS of horses experimentally and naturally infected with S. neurona [64].

2.9. Diagnosis

2.9.1. General Information

Currently, there is no antemortem test that provides definitive evidence of disease. The most rewarding diagnostic tool and the “gold standard” for diagnosis of EPM is postmortem immunohistochemistry of the gray and white matter. Often times, this is an unacceptable route of diagnosis in a clinical setting. Antemortem diagnosis is typically presumptive and based on clinical signs that are consistent with EPM, laboratory testing, and response to therapy. Clinical signs are often indistinguishable from those of other neurologic diseases, such as cervical stenotic myelopathy, equine degenerative myelopathy, or equine herpes virus infections. Response to therapy may be a relatively accurate diagnostic tool, although the time delay can be frustrating for both owners and practitioners.

General laboratory evaluation is rarely helpful in diagnosing EPM. Horses affected with EPM usually have normal peripheral complete blood counts and serum chemistry profiles [38].

2.9.2. CSF indices

The diagnosis of EPM generally relies on evaluation of cerebrospinal fluid. Either a standing lumbosacral aspirate or an atlanto-occipital aspirate under general
anesthesia can be performed [6]. EPM does not cause consistent changes in the CSF’s color, clarity, cell counts or concentrations of protein, glucose or antibody of infected horses [38]. Several CSF indices, including the albumin quotient and the immunoglobulin G index, have been used to differentiate antibodies resulting from peripheral blood contamination during CSF aspiration and intrathecal production. Neither index, or their combination, were determined clinically useful due to their wide variability in normal and affected horses [65, 66]. In addition, increases in CSF red blood cell concentration that may cause a positive result using other diagnostic tests (i.e. Western blot) may not be drastic enough to alter the albumin quotient or IgG index [65].

Njoku et al demonstrated lower levels of nitric oxide (NO) metabolites in the CSF of horses with clinical EPM. Measuring NO metabolites could feasibly improve the accuracy of other diagnostic tests if used in combination [64].

2.9.3. **Indirect fluorescent antibody titer**

The first antemortem test available for the diagnosis of EPM was the indirect fluorescent antibody titer (IFAT) that measured a sample’s antibody reactivity to *S. cruzi*. The downside of the test was its inability to distinguish between antibodies specific to *S. neurona* and antibodies specific to *S. cruzi* [67]. More recently, Duarte et al compared the performance of the IFA with the Western blot and modified Western blot. The IFA test was demonstrated to be more accurate than both immunoblots using serum, as well as being identified as less expensive and easier to perform [68]. Still, problems persist with this diagnostic tool in that infections with other *Sarcocystis* spp may cause false-positive results [68, 69].
2.9.4. Immunoblots

Western blot is an antibody-based test that detects IgG specific for *S. neurona* in either the serum or CSF. The Western blot is an immunoblot test that was developed by Granstrom et al and became available in 1993 [67]. The goal during test development was to find specific antigens unique to *S. neurona*, which could be identified by their unique pattern for disease diagnosis. Eight proteins were detected by *S. neurona* antiserum from EPM-infected horses, and laboratories vary slightly on the band patterns used for diagnosis [67]. It has gained wide acceptance and is one of the only antemortem tests available for EPM at this time. Ideally, a paired serum and CSF sample should be tested, because seroconversion alone does not confirm active disease. The test is readily accessible and has excellent sensitivity and specificity as a measure of exposure [68, 70].

The test’s downfall is its specificity for identifying active disease. A recent report identified that specificity was only 44% for horses with neurologic abnormalities and 60% for horses without, which indicates a high rate of false positive test results [70]. Obviously, those positive results should be carefully interpreted in light of disease prevalence and the clinical picture. Causes of false positive results include iatrogenic contamination of the CSF with peripheral blood in seropositive horses, increased permeability of the blood brain barrier of seropositive horses with another neurologic disease, subclinical *S. neurona* infection combined with another neurologic disease, or cross-reactivity in horses infected with *S. neurona*-like organisms [70]. In addition, immunoblot tests in general are subjective and lack the ability to quantify antibody production.
More recently, a modified Western blot was reported as having a sensitivity and specificity approaching 100% when it evaluated serum from culture positive horses [71]. The modification involves pretreatment of antigens with purified bovine IgG from animals with high titers against *S. cruzi*. The theory is that antigens common to *S. neurona* and *S. cruzi* would be blocked by the bovine IgG, and only the protein not common to *S. cruzi* would be recognized. This blocking of the nonspecific proteins would ideally improve immunoblot interpretation. Many EPM researchers disagree with the modification, and more thorough evaluation will be necessary.

With a reported seroprevalence of infection with *S. neurona* being higher than 50% in many regions [72-75], being able to classify groups into exposed and diseased is crucial. Although the Western blot test is useful for ruling out EPM when the prevalence of infection is low [70], the Western blot is an insufficient solitary diagnostic tool and likely causes overestimation of diseased horses and inappropriate treatment of horses without active *S. neurona* infections.

2.9.5. Polymerase Chain reaction

A polymerase chain reaction test for *S. neurona* antigen DNA in CSF was developed, but had a low sensitivity and therefore was not clinically useful [76]. PCR is typically an extremely specific tool, but its sensitivity may have been hindered by the intracellular nature of *S. neurona*, the inability to identify the antigen’s DNA in a biological sample, or rapid destruction of the DNA.

2.9.6. Clinical Signs

EPM is considered a progressively debilitating disease affecting the CNS of horses, and causing acute to slowly progressive, focal or multifocal neurologic deficits [1,
3, 4, 16, 38, 77]. The disease can be seen in any age animal, although the most common age group affected are 1-4 year olds [78]. Clinical signs often involve one or more limbs and result in weakness, stumbling, ataxia, spasticity, and sometimes muscle atrophy.

Cranial nerve deficits and seizures are less common, but can occur [77-79]. In Saville et al.'s review of risk factors associated with EPM, 11.6% of horses had cranial nerve disease and 6.0% had a history of seizures [78]. The common clinical signs associated with brain lesion include vestibular ataxia, facial paresis and dysphagia. There may also be areas of hypoalgesia or sensory loss, and depending on the brain and brainstem involvement, there may be mild depression [77]. Factors governing severity of the clinical signs are unknown, but the variability of clinical signs may be a result of the organism's ability to affect white and gray matter randomly and at many sites. Typically, the horse is systemically stable and has normal vital parameters.

2.10. Therapy

2.10.1. General Information

Treatment of affected horses should begin immediately after a presumptive diagnosis is made, and sooner if a delayed diagnosis is expected. Continuation of active infection may lead to further CNS damage and worsening of clinical disease. Estimations of 60-70% of horses respond to treatment for EPM, while untreated horses may progress to recumbency or death [78]. There is also evidence to suggest that horses can clear the organism without therapy, which would help explain the population of normal horses with CSF antibodies to S. neurona [41]. However, according to Saville et al, horses that were treated were ten times more likely to improve than their counterparts that were not treated, and interestingly, races horses were more likely to improve with therapy than
horses used for breeding. Also, horses with milder neurologic disease at the time of diagnosis were more likely to improve than those with severe disease [78].

2.10.2. Pyramethamine

Anti/protozoal drugs are an obvious choice for *S. neurona* infections. Originally, pyramethamine (Daraprim®, Burrows-Wellcome), which is a diaminopyrimidine that binds the enzyme dihydrofolate reductase and prevents folate metabolism, was the treatment of choice in combination with sulfadiazine. Protozoa must synthesize their own folic acid, whereas mammalian cells use preformed folic acid. Pharmacodynamics and pharmacokinetics studies have established a dosing regimen of 1mg/kg/day orally as the recommended therapy [80]. This combination often requires an extended treatment plan, which may last three to four months. Possible side effects of pyramethamine include leucopenia, thrombocytopenia, diarrhea; teratogenic effects in foals of treated mares; and decreased reproductive performance in stallions [81, 82]. Pyrimethamine can have some activity against the mammalian enzyme, so supplemental therapy with folinic acid may prevent toxic effects of the drug [83]. Administration of folic acid, especially in pregnant mares, is not recommended as that it can result in a worsened folic acid deficiency and cause subsequent skin abnormalities, anemia, bone marrow suppression, or developmental abnormalities in their foals [81].

2.10.3. Triazines

Triazines are a class of anti/protozoal drugs which are more selectively toxic for *S. neurona* by acting on a structure (plastid body organelle) that is not present in vertebrate cells [84-86]. Triazines that have been used for the treatment of EPM in the horse include toltrazuril (Baycox®, Bayer Inc), ponazuril (Marquis®, Bayer Inc) and
diclazuril (Clinicox®, Schering-Plough Animal Health Corporation). Ponazuril is currently the only drug in this class licensed for use in the treatment of EPM.

Diclazuril is triazine effective against a broad range of Apicomplexan parasites. Preliminary pharmacokinetic analysis and clinical trials reported adequate peak serum concentrations and clinical efficacy, respectively [84, 87]. Unfortunately, the only available form of the drug is as a poultry feed additive (Clinicox®), and requires a very large oral dose, which many horses will not voluntarily consume. More recent studies have evaluated diclazuril as a possible preventive agent [88].

Toltrazuril is another triazine agent with broad-spectrum anti/protozoal effects, whose active metabolite is ponazuril. The drug was found to have adequate CSF penetration and concentrations, as well as cause only mild side effects, including mild colic and depression [77]. Clinical trials have not been performed to this author’s knowledge, but its metabolite, ponazuril, is currently one of two licensed drugs for the treatment of EPM.

Ponazuril (Marquis®) is likely one of the most commonly used treatments for EPM. Efficacy trials of oral ponazuril (5mg/kg or 10mg/kg once daily for 28 days) established success rates, defined as improvements in neurologic score by at least once grade or conversion to negative status on Western blot by 90 days post-treatment, of 62% in the horse [89]. In general, improvements were seen during the 28 treatment period, although a portion of horses continued to improve after treatment has been discontinued. Participants did not report any adverse affects and compliance for administration was considered good. This study excluded very aged horses, horses less than 6 months old, and pregnant mares or horses used for breeding. In a study by the same group of
investigators, the pharmacokinetics of oral ponazuril was investigated. By 7 days after therapy began, and throughout the 28 day treatment period, the concentration of ponazuril in the CSF were presumed to be adequate for the treatment of EPM [90].

Finally, in a study evaluating the safety of oral ponazuril, few side effects were seen during therapy. At necropsy, three of the four mares treated with high dose (30mg/kg) had uterine edema [91]. Further studies to determine the safety of this pharmaceutical in pregnant or breeding animals are necessary.

2.10.4. Benzamides

Nitazoxanide (Navigator®, Idexx) is a 5-nitrothiazole benzamide compound that has efficacy against a wide variety of parasites, including protozoa. It is not an uncommon therapy for use of intestinal parasitism in humans [92, 93]. In a study by McClure et al, horses treated with nitazoxanide (50mg/kg orally once daily for 28 days) showed improvement in their neurologic signs, although did not convert to negative results on their Western blots [94]. Side effects of nitazoxanide can be severe and include fatal colitis and laminitis. In toxicity studies, anorexia, depression, and diarrhea with some deaths were observed in horses fed two to eight times the recommended dosage [77, 95]. Currently, the manufacturer recommends feeding with a fatty meal to encourage absorption in the small intestine and prevent traveling to the large colon, and owners are given strict dosing and monitoring regimens to follow.

2.10.5. Adjunct therapies

Other treatments for EPM, and many neurologic diseases in general, include corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). Corticosteroids should be used with hesitation with an infectious disease, as their use can cause
immunosuppression and possibly exacerbate the disease process. Although historically, corticosteroids were given to horses with EPM to increase the number of parasites at post-mortem [5, 96], several studies have associated the use of dexamethasone with less severe clinical signs [40, 41]. NSAIDs, as well as corticosteroids, may help with the CNS inflammation that is likely associated with the clinical signs. Dimethylsulfoxide (DMSO), an anti-inflammatory and free radical scavenger, is used commonly in horses with neurologic disease with anecdotal success [38]. Finally, because the CNS is so susceptible to oxidant damage, supplemental therapy with vitamin E has been recommended. Although efficacy trials have not been performed on vitamin E, it is unlikely to cause harm at the recommended dosage [77].

Immune stimulation with products such as Equimune (Vetrempharm, Canada) and levamisole phosphate (Levasole®, Schering-Plough) have had anecdotal success as additional therapies for EPM [97]. To this author’s knowledge, efficacy trials looking specifically at horses with EPM have not been performed.

2.11. Prevention

2.11.1. Chemoprophylaxis

At this time, there are no pharmaceutical agents commercially available for the prevention of EPM. Diclazuril has been evaluated as both a prevention and treatment. In a study by Dubey et al, gamma interferon KO mice were challenged with *S. neurona* sporocysts, and the mice were either treated with diclazuril prior to inoculation or starting at varying times post-inoculation. The mice treating before and up to 7 days post-infection did not develop disease or histopathologic changes consistent with EPM [88]. This study supports that diclazuril may not only be a treatment option, but a prophylactic
option as well. In addition, pharmacokinetic work on diclazuril showed good maintenance of plasma and CSF concentrations after oral administration [87].

2.11.2. Vaccination

Currently, Fort Dodge Animal Health has a conditional licensed vaccine to prevent EPM. At the time of this writing, there are efficacy trials being performed using the EPM equine model established by researchers at The Ohio State University (Saville, pers comm.). The vaccine has been shown to stimulate antibody production, which can be detected in the serum or CSF [98, 99]. In these limited evaluations, no adverse side effects were seen in pregnant or non-pregnant animals. Currently, the vaccine has no claim of safety in reproductive animals.

2.12. Management

There are no proven therapeutic preventive measures for EPM yet identified, which leaves an owner with management adjustments. Prevention techniques for EPM are limited to eliminating access of opossums and other wildlife to feed and water in the horse’s environment. In addition, reducing stress and regular, moderate exercise may be helpful in preventing disease [100].

2.13. Epidemiology

EPM is a disease first identified in Kentucky in the 1960s [2]. Since that time, it is known to cause neurologic disease throughout North and South America [77]. There have also been seropositive and affected horses identified in Europe and Asia, although the common history is importation or visitation to the US [17, 77, 101-105]. One article by Pitel et al reports on 28 horses in France with EPM-like disease and positive serum
and CSF Western blot results, of which only 2 horses were born or stayed in the US [105].

Most clinical studies of EPM report sporadic infection versus multiple horses from the same property, however “outbreak” situations have been described [103, 106].

2.13.1. Risk Factors

In Saville et al’s review of risk factors associated with development of EPM, and clinical improvement and survival of horses with EPM, approximately 65% of treated horses improved, and 55% of horses survived. Not surprisingly, horses that improved with therapy were 50 times more likely to survive than horses that did not improve. This figure likely reflects several variables, including owner finances, value of the horses and cost of therapy. Survival rates were higher for treated animals with more mild clinical signs, then for treated horses with more severe signs. As mentioned previously, horses that were treated for EPM were 10 times more likely to improve than horses that were not treated, and horses used primarily for racing were 9 times more likely to improve after treatment, compared with breeding animals [78, 107].

Risk factors for developing EPM included age (1-5 years; greater than 13 years), season of hospital admission (spring, summer, fall), prior diagnosis of EPM on the same property, opossums on the premises, unsecured feed containers, health problems or a stressful event prior to admission, and racing or showing as a primary use [107]. In addition, the National Animal Health Monitoring System (NAHMS) identified an even higher risk of developing EPM if opossums were seen frequently, if there were a high number of horses on the property, purchased versus home-grown grain, use of wood chips or shavings as bedding, presence of rats or mice, and a high human population
density. A decreased risk was identified if there were woods within 5 miles of the
premises and where surface water was the primary drinking source [108].

2.13.2. Seroprevalence of S. neurona in horses

Seroprevalence studies have demonstrated that in many areas, over half of the
horse population has been exposed to S. neurona. Seroprevalance represents exposure to
S. neurona and not the presence of active disease. Recent studies using the Western blot
have shown seroprevalence in Ohio at 53% [75], central Wyoming at 6.5% [37],
Oklahoma at 89.2% [73], southeastern Pennsylvania at 45.3% [74], and Oregon at 45%
[72].

2.13.3. Seroprevalance of Neospora

The seroprevalence Neospora species in horses is less studied, however
antibodies to N. caninum were found in 31.1% of horses in central Wyoming [37], 23.3%
of horses from meat packing plants in Nebraska and Texas [109], and approximately 11%
in Alabama [110] and Missouri [111]. In contrast, Dubey et al and Gupta et al found a
0% seroprevalence rate in horse sera from Brazil and Korea, respectively [112, 113]. The
relevance of seroconversion to Neospora spp. in relation to clinical EPM is unknown.

2.13.4. Seroprevalence of S. neurona in opossums

The North American opossum (Didelphis virginiana) is the definitive host for
several species of Sarcocystis, including S. neurona. Several different studies have
reported on the prevalence of S. neurona sporocysts in opossums, and rates have varied
from 8/44 (18%) to 19/72 (26%) to 2/21 (28%) depending on the method of detecting
infection and the area of the country [20, 114, 115]. Risk factors associated with
opossum infection with S. neurona included spring time and good body condition [116].
2.13.5. Disease Incidence

In contrast to seroprevalence, the incidence of disease is very low, and was estimated by the NAHMS to be approximately 0.14% or 14 ± 6 cases per 10,000 horses per year [108]. The incidence in horses, in increasing frequency, was pleasure horses, breeding horses, racing horses and competition/show horses.

2.14. References


35


CHAPTER 3

Development and evaluation of a *Sarcocystis neurona*-specific IgM Capture Enzyme Linked Immunosorbent Assay.

3.1. Abstract

Equine protozoal myeloencephalitis (EPM) is a serious neurologic disease of horses caused primarily by the protozoal parasite *Sarcocystis neurona* [1]. Currently, antemortem testing is limited in its specificity in exposed and diseased horses [2]. This paper reports on the development of an IgM capture enzyme linked immunoassay (ELISA) for the identification of acute infection with *S. neurona*. The ELISA was based on the *S. neurona* low molecular weight protein SNUCD-1 antigen and the monoclonal antibody 2G5 labeled with horseradish peroxidase [3]. The test was evaluated using serum and CSF from 12 horses experimentally infected with 1.5 million *S. neurona* sporocysts and 16 horses experimentally infected with varying doses (100 to 100,000) of *S. neurona* sporocysts, all of whose histopathology results were available.

The hypothesis of this study was that serum and cerebrospinal fluid (CSF) of horses experimentally challenged with *S. neurona* would have an increased *S. neurona*-specific IgM (Sn-IgM) concentration relative to their pre-infected state. For horses challenged with 1.5 million sporocysts, the results indicate a significant increase in serum
Sn-IgM concentrations relative to pre-inoculation for weeks 2 through 6 post-inoculation (P-value <0.0001). For horses inoculated with lower doses of S. neurona, there were significant increases in Sn-IgM concentration at various points in time depending on the inoculation dose (P-value< 0.01). Finally, there was a significant increase between the pre- and post-inoculation CSF Sn-IgM concentrations (P-value < 0.0001). Our results support the assay as a valuable addition as a diagnostic tool during the acute phase of EPM.

3.2. Introduction

Equine Protozoal Myeloencephalitis is a serious neurologic disease caused primarily by Sarcocystis neurona [1, 4]. It is considered a progressively debilitating disease causing acute to slowly progressive, focal or multifocal neurologic deficits [5]. Currently, the gold standard diagnostic test for EPM is postmortem immunohistochemistry of the gray and white matter. Western blot is an antibody-based test that detects IgG specific for S. neurona in equine serum and CSF, and is one of the only antemortem tests presently available. The test has excellent sensitivity, but its downfall is its specificity for identifying active disease [2, 6]. A recent report identified a specificity of only 44% using CSF from horses with neurologic abnormalities and 60% for horses without; while a follow-up study using serum from a subset of the same population of horses reported a specificity of 87.2% [2, 6]. With a reported seroprevalence of infection with S. neurona higher than 50% in some regions [7-9], being able to classify animals as exposed or diseased is crucial. Western blot is an insufficient diagnostic tool and likely causes overestimation of diseased horses and inappropriate treatment of horses without active S. neurona infections.
Activation of humoral immunity by a primary exposure to intracellular parasite antigen initiates a strong IgM response in other Apicomplexan organisms [10-12]. Primary infections with *Sarcocystis* species in intermediate hosts show peak IgM responses at approximately 35 days for sheep and pigs, and 3-4 weeks for cattle [11-13]. In sheep, IgM responses were higher with larger sporocyst doses, suggesting that antibody development may be related to the level of exposure [13]. In addition, in a challenge model in which immunized pigs were exposed to *S. miescheriana*, there was a secondary rise in IgM titers, suggesting an anamnestic response after re-exposure that was not seen in either cattle or sheep [12, 13].

In a review of specific antibody responses in pigs experimentally infected with *Toxoplasma gondii* tachyzoites and oocysts orally, there was a serum IgM response that appeared by seven days and peaked at ten days post-infection [14]. The increased IgM concentrations were no longer distinguishable from the background reactions by day 21-24 after infection, making evaluation of the short-lived IgM response a potential for the acute phase of the infection [15, 16].

In general, serum and CSF IgM testing is reserved for the identification of acutely infected individuals. Intrathecal IgM is a useful diagnostic tool because of its antigen specificity and its large size and unlikelihood of crossing the blood brain barrier [17, 18]. Its usefulness for chronic or repeat infections depends on the persistence of significant IgM concentrations and the robustness of the IgM response in subsequent infections, respectively. The purpose of the present study was to develop an *S. neurona*-specific IgM capture ELISA and evaluate its usefulness in detecting acute EPM infections in naïve individuals using either serum or CSF.
3.3. Materials and Methods

3.3.1. Collection of *Sarcocystis neurona* sporocysts, preparation of inoculums, infection of horses, collection of samples and necropsy results

Collection of *S. neurona* sporocysts, preparation of inoculums and infection of horses was completed in two previous studies and the stored serum and CSF samples are part of a collaborative pool of samples at The Ohio State University [19, 20]. All pre-infected experimental horses were seronegative by Western blot, came from an area of Canada in which EPM has never been reported, and had no evidence of neurologic disease at the time of inoculation. Although variable, the dose of *S. neurona* sporocysts, neurologic deficits, serum and CSF conversion results, and histopathology results for each infected horse are reported in those studies.

3.3.2. Experimental Samples

A total of 12 horses (Group 1) that were experimentally infected with 1,500,000 sporocysts had serial serum and pre- and post-inoculation CSF samples collected [20]. Six horses (Group 1A) (7, 8, 12, 13, 14, and 20) had serum samples collected weekly from week 0 to week 4; these six horses were not subjected to transportation stress. Six horses (Group 1B) (3, 11, 23, 24, 27, and 28) had serum samples collected weekly from week 0 to week 7; these six horses were subjected to transportation stress at 18 days post-inoculation. For all samples, post-inoculation CSF collection was obtained from the lumbosacral location immediately prior to euthanasia.

A total of 16 horses (Group 2) (B4, B5, B18, B41, C12, C14, C 19, C37, D8, D13, D 21, D23, E10, E11, E17, and E30) with sporocyst doses of 100 sporocysts/inoculum (Group 2B), 1000 sporocysts/inoculum (Group 2C), 10,000 sporocysts/inoculum (Group
2D) and 100,000 sporocysts/inoculum (Group 2E) had serum collected weekly from week 0 to week 4 [19]. Although CSF samples were collected during that study, there were no remaining samples available for the use in the present study.

3.3.3. **West Nile Virus-positive Samples**

Seven WNV-positive serum samples were supplied by the Ohio Department of Agriculture (ODA). These samples were from acutely neurologic horses that tested positive using the WNV-specific IgM capture ELISA (MAC-ELISA). Information on whether these horses were tested for EPM or subsequent results of any testing was unknown. This group was used for the purpose of looking for obvious cross-reactivity with WNV antibodies.

3.3.4. **Single Radial ImmunoDiffusion (SRID)**

Total equine IgM was measured by SRID in pre-inoculation and 3 week post-inoculation serum samples from Group 1A using a commercially available test (VMRD, Pullman, WA). The 3 week post-inoculation point-in-time was chosen based on results of the WNV MAC-ELISA and peak IgM levels being attained at approximately the same time [21]. The SRID identified whether IgM was present in the samples, and also provided a generalized assessment of the magnitude of each horse’s IgM response to the *S. neurona* inoculation.

3.3.5. **Preparation of *S. neurona* Antigen**

*Sarcocystis neurona* (SN-UCD1) protozoa were grown in equine dermal (ATCC, CCL57) monolayers in RPMI-1640 media supplemented with 10% v/v heat inactivated fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 5×10⁻² mM 2-mercaptoethanol (2-ME), 50 units penicillin/mL, and 50 μg streptomycin/mL, and
incubated at 37°C. At approximately 75% host cell infection or lysis, parasites were harvested, washed, counted, and immediately frozen at -80°C.

The *S. neurona* parasite pellets underwent hypotonic lysis followed by 3 rapid freeze thaw cycles to disrupt intact merozoites; antigen was diluted to the appropriate concentration for ELISA testing using 1% bovine serum albumin in phosphate-buffered saline (PBS, pH 7.3) with 0.5% Tween 20 (PBST). The average antigen concentrations used for the ELISA was approximately 4.0 x 10⁴/μl.

3.3.6. Immunoblot

Pre- and post-experimentally infected horse samples from Group 1A were evaluated for *S. neurona*-specific IgM responses using an immunoblot to examine the antibody-antigen protein reactions (based on the epitope specificity of the MAb 2G5-2 HRP). parasite antigen (SN UCD1) and loading dye with or without 10% 2-mercaptoethanol were heated at 70°C for 10 minutes; 4-12% NuPage BisTris Zoom Gels (Invitrogen, Carlsbad, CA) were used for protein separation by electrophoresis followed by electrotransfer to nitrocellulose membranes. The blots were probed with sera from positive and negative control dilution samples.

3.3.7. ELISA reagents

Goat anti-horse IgM is a commercially available antibody (Kirkegaard & Perry Laboratories KPL, Gaithersburg, MD). Monoclonal antibody 2G5-2 underwent a chemical covalent linkage using a commercially available peroxidase labeling kit (Roche Molecular Biochemicals, Indianapolis, IN) following manufacturer’s recommendations. Following the labeling reaction, the antibody underwent dialysis to remove excess peroxidase and to return the MAb 2G5-2 to a physiologic pH to avoid denaturation.
Immunoblots were performed in order to confirm labeling and retained activity of the antibody against specific *S. neurona* antigen.

3.3.8. **ELISA preparation**

Optimal dilutions were established using checkerboard titrations with dilutions of coating preparations, sera and CSF, monoclonal antibodies and substrates. Ninety-six well ELISA microtiter plates (Falcon, Lincoln Park, NJ) were coated overnight at 4°C with Goat anti-equine IgM antibody at 1:100 dilution (1000 ng/well) in a sodium carbonate-bicarbonate coating buffer (0.015 M Na₂CO₃, 0.03 M NaHCO₃, and 0.003 M NaN₃, pH 9.6) for a total volume of 100 µl/well. After overnight incubation, the antibody was removed. Plates were incubated with 1% bovine serum albumin (BSA) in PBST for one hour at 37°C with an initial 5 minutes of micromixing. Serum at 1:100 dilution with PBST or CSF at 1:2 dilution with PBST was added to the appropriate wells in duplicate. The plates were micromixed for five minutes and incubated at room temperature for one hour. Plates were washed three times with PBST. SNUCD-1 antigen was added to each well for a total volume of 100 µl/well. Plates were micromixed for five minutes, incubated at 37°C for one hour and room temperature for one hour. Plates were washed three times with PBST. Monoclonal antibody (MAb) labeled horseradish peroxidase (HRP) diluted to 1:250 with 1% BSA in PBST was added to each well for a total volume of 100 µl/well. Plates were micromixed for five minutes and incubated for one hour at room temperature in the dark. Plates were washed three times with PBST. Color development was achieved using 2’, 2’-azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS, Kirkegaard & Perry Laboratories) and optical densities were determined using an ELISA plate reader (MTX Labsystems, Vienna, VA) at 405 nm.
3.3.9. **Statistical Analysis**

A percent positivity (Equation 1) was determined and utilized in order to establish a relative measurement. Using this technique, as described by Wright et al, each sample was compared to the positive and negative control within their testing group (plate) and a percent positive (PP) was determined [22]. The PP represents the samples percent of the positive control, therefore creating a relative OD value and not relying solely on an individual OD level to identify a positive or negative result. Creating a PP value will not change the outcome of the test (i.e. whether the result is positive or negative), but merely create an interpretable value for use at multiple laboratories [22].

Statistical analyses were performed using Stata 7.0 (Stata Corporation, College Station, TX) and SAS 9.1 (SAS Institute, Cary, NC). Pre- and post- inoculation SRID results and differences were summarized using means and ranges for six experimental and seven WNV-positive horses. For the ELISA data, mean and standard deviation of OD values for positive and negative controls were calculated. For each experimental sample a single mean PP value was determined each week. For horses in Group 1 and 2, the week at which PP peaked was determined; the mean week of peak PP was calculated separately for Groups 1, 2B, 2C, 2D, and 2E. Also, for Groups 1, 2B, 2C, 2D, and 2E, a one way repeated measures ANOVA was performed with time post-inoculation as the only factor to determine if mean PP value differed between weeks. Experimental CSF pre- and post- inoculation were compared for Group 1A and Group 1B using a one way repeated measures ANOVA with time post-inoculation as the only factor to determine if mean PP of CSF differed over time. If any of the ANOVA analyses indicated that weekly mean PP values were significantly different at a P-value <0.01, then all post-hoc
pairwise comparisons of mean PP values were made using the Tukey-Kramer adjustment for multiple comparisons. Additionally, OD and PP values for experimental negative control and WNV-positive samples were compared using a t-test.

Finally, for Group 1 horses, the correlation between neurologic score and serum PP value was compared using Spearman's Correlation Coefficient (ρ). This was performed for all observations, as well as within each horse and within each week.

3.4. Results

3.4.1. Experimental Samples

All 28 horses except for one (E17) developed neurologic deficits after inoculation with *S. neurona* sporocysts. Neurologic deficits developed within 7 to 29 days after experimental challenge and were most consistently dramatic in the groups receiving larger inoculate doses. All horses except for three (B5, B41, D21) seroconverted using Western blot (IgG) technique; and the same 25 horses converted using Western blot in the CSF. No parasite was recovered from the CNS of any horse in either of the two studies [19, 20].

3.4.2. Single Radial Immunodiffusion

SRID evaluation was performed on the pre- and 3-week post-inoculation serum from Group 1A, as well as on the serum of seven WNV positive horses. The average pre-inoculation IgM concentration of all the experimental samples was 108.2 mg/dL (range: 58.4 – 141.4 mg/dL); the average post-inoculation IgM concentration was 137.0 mg/dL (range: 87.3 – 172.8); and all horses had an increase in IgM concentration with an average increase of 28.8 mg/dL (range: 4.6 – 57.2 mg/dL). The WNV positive horses had an average IgM concentration of 208.3 mg/dL (range: 65.9 – 435.4 mg/dL).
Reference value concentrations of IgM in normal, fit horses were previously reported as 103 ± 40 mg/dL by Perkins et al [23].

3.4.3. Immunoblots

Immunoblots were performed using *S. neurona* parasite antigen (SN-UCD1) on pre- and 3-week post-inoculation serum samples from Group 1A. In addition, known EPM negative and EPM positive control samples were included in the immunoblots. The post-inoculation samples demonstrated increases in Sn-IgM antibodies indicated by a dramatic increase in reaction intensity to the antigen (Figure 3.1).

3.4.4. Enzyme Linked Immunosorbent Assay

Each plate had duplicate blank, positive control and negative control wells. The mean OD values obtained for the positive and negative controls across all plates were 1.502 ± 0.483 and -0.056 ± 0.374, respectively.

3.4.5. Experimental Serum Samples

Group 1A serum samples were evaluated at all dates collected, which represented weeks 0, 1, 2, 3 and 4. There was an initial trend of increasing Sn-IgM concentrations with an average peak PP measurement at week 2.8 (range: week 2-4). There was a significant increase between the measurement at week 0 and the measurements at weeks 2, 3, and 4 (P-value <0.0001). Group 1B serum samples were evaluated at all dates collected, which represented week 0, 1, 2, 3, 4, 5, 6, and 7. There was a similar initial trend of increasing Sn-IgM concentrations with an average peak PP measurement at week 2.3 (range: week 2-4). There was a significant increase between the measurements at week 0 and the measurements at weeks 2, 3, 4, 5, and 6 (P-value <0.0001). There was no significant difference between week 0 and week 7 (P-value = 0.096) (Figure 3.2).
Serum samples from Groups 2B-2E were evaluated at weeks 0, 1, 2, 3, and 4. There were significantly different PP values between week 0 and week 4 for Group 2B (P-value = 0.0075); between week 0 and weeks 3 and 4 for Groups 2C (P-value <0.001) and 2D (P-value <0.01); and between week 0 and weeks 2, 3 and 4 for group 2E (P-value <0.0001) (Figure 3.3).

3.4.6. Experimental CSF Samples

Group 1A and Group 1B CSF samples were evaluated at pre-inoculation (week 0) and post-inoculation (week 4), and pre-inoculation (week 0) and post-inoculation (week 7), respectively. These data are shown in Figure 3.4. There was a significant increase between the pre- and post-inoculation PP values within each group (P-value < 0.0001). There was also a significant difference between the post-inoculation values of each group, with the CSF samples collected at week 4 (Group 1A) having higher PP values than those collected at week 7 (Group 1B) (P-value = 0.0001).

3.4.7. West Nile Virus-positive Samples

The seven WNV samples had an average OD value of -0.117 ± 0.193; and average PP value of 3.09 ± 6.42%. These data are shown in Figure 3.5. There was no statistical difference between the negative control samples and the WNV samples (P-value = 0.5802).

3.4.8. Correlation between IgM concentration and neurologic signs

The overall Sn-IgM concentrations, as reflected by the calculated PP values, had a weak linear correlation with the severity of neurologic signs in our population of samples challenged with the same dose of *S. neurona* sporocysts (r=0.4682) (Figure 3.6). However, at any given week, there is little correlation between PP and neurologic scores.
This suggests usefulness in following a horse over time and multiple PP values; however, a single PP measurement would unlikely correlate with the neurologic score, and it would be difficult to predict a PP value from a neurologic score without a "reference point" for both values.

3.5. Discussion

Equine protozoal myeloencephalitis is a disease with a high seroprevalence and a low incidence of disease, making it a diagnostic challenge [2, 24-26]. In this study, we were able to detect significant differences in Sn-IgM concentrations between the pre-inoculation and post-inoculation serum and CSF samples. Typically, there was a significant increase from baseline in serum Sn-IgM concentrations by 2 weeks post-inoculation, which is similar to the IgM immune response kinetics in other Apicomplexan infections [12, 14, 15]. Serum Sn-IgM concentrations peaked between weeks 2 and 4. Although not significant, horses receiving smaller inoculate doses of S. neurona appeared to have Sn-IgM concentrations which peaked later compared with horses inoculated with larger doses of S. neurona. This may suggest that the kinetics of the antibody response is related to the level of exposure. Interestingly, the timing of seroconversion using Western blot had a linear relationship to the dose of sporocyst received [19]. Unfortunately, the persistence of the Sn-IgM response as a function of dose was not evaluated due to the limited time for which serum samples were collected.

Statistically significant differences in serum Sn-IgM concentrations persisted for up to 6 weeks in horses receiving a high dose of sporocysts, and were not different from baseline by 7 weeks after inoculation. This likely reflects the normal waning of a primary IgM response over time, and unfortunately, the long-term persistence of IgM was
not evaluated due to the limited sample collection dates available. At this time, the 
author is unaware of any studies elucidating the kinetics of the IgM response within 
horses experimentally or naturally exposed to S. neurona. However, a measurable 
increase in Sn-IgM concentrations for over a month would provide a clinically relevant 
testing period in acutely affected horses.

Pre-inoculation CSF samples were consistently low and post-inoculation CSF 
samples were significantly higher for all 12 samples available. Interestingly, the post-
inoculation Sn-IgM concentration was higher in the group of horses euthanized 4 weeks 
after challenge than those euthanized at 7 weeks after challenge. This likely indicates 
that intrathecal IgM antibody concentrations begin to decrease between 4 and 7 weeks 
following infection with S. neurona, which is similar in other diseases in which 
intrathecal IgM concentrations are monitored, such as tick-borne encephalitis [27]. 
Because there was only a single post-inoculation sample of CSF from each horse, it is 
impossible to identify the point at which Sn-IgM concentration peaks or when it would 
become statistically insignificant. In summary, both the post-inoculation serum and CSF 
samples have dramatic increases in Sn-IgM concentration that persist for over one month 
as detected by the S. neurona-specific IgM ELISA, which indicates a usefulness as a 
diagnostic tool for detecting acute EPM.

Serum and CSF samples used for this study were collected during previous 
projects evaluating equine models for EPM [19, 20]. Inconsistencies between horses 
within similar groups in their degree of neurologic deficits, serum and/or CSF Western 
blot conversion or histopathology findings may be attributed to insufficient dose to 
establish an infection or variations in individual immune responses [19]. In our study,
there was a weak correlation between PP values and neurologic score over all observations; however a relationship was not detected on a week to week basis. Therefore, although the trend of increasing PP values with increasing severity of neurologic signs was visible, a single measurement was not useful in predicting a PP value. In addition, the variation of neurologic signs between horses makes using neurologic deficits to predict PP value impossible.

West Nile virus positive horses were used to evaluate the *S. neurona*-specific IgM capture ELISA for obvious cross-reactivity with another commonly diagnosed neurologic disease of horses. Using SRID, the WNV positive sera had high levels of non-specific IgM, which was not surprising due to the acute nature of infection and their positive MAC-ELISA results. No cross-reactivity was identified between the WNV samples and *S. neurona* experimentally infected samples using the *S. neurona*-specific IgM capture ELISA. The influence of potentially cross-reacting antibodies induced by other infections could be further evaluated.

A diagnostic test is only as valuable as its ability to produce consistent results with tolerable standard errors between multiple testing trials. Because of inherent variations between laboratories and assays, results are better expressed in relative rather than absolute values. Advantages of utilizing the PP value as a reflection of the Sn-IgM concentration is that it corrects for inter-laboratory differences in the background activity [22].

Animals with known experimental exposure to *S. neurona* and known clinical disease are the ideal population in which to develop a diagnostic test, although they do not reflect the population to which the test will be applied. Experimentally infected
horses are inoculated with a very large dose of antigen and may have a more robust IgM response then naturally exposed animals. Additional testing utilizing field samples would further elucidate on the clinical usefulness of the Sn-IgM capture ELISA. In addition, a series of vaccinated horses could be evaluated to determine if there is cross reaction of antibodies induced by vaccination with the assay. Typically, vaccinations do not stimulate persistent IgM responses, although there may be variation depending on the vaccine type and the disease [28, 29]. Any Sn-IgM response to vaccination with the killed S. neurona vaccine has not been reported to this author’s knowledge.

As indicated by the enormous population of seropositive horses in certain areas of the country, a significant portion of the horse population has at least one exposure to S. neurona [8, 25, 26, 30]. Previously exposed animals may have variations in their antibody response in subsequent infections. Further studies designed to elucidate both the persistence of Sn-IgM over time and the Sn-IgM response in re-exposed animals will better establish the value of this assay as a diagnostic tool. Equine protozoal myeloencephalitis is an incompletely understood disease that can cause devastating neurologic deficits in horses. Currently, little is known about the pathophysiology of the disease, its course within the horse, and the role of the horse’s immune response. In addition, there is a paucity of accurate diagnostic tools available for EPM. Identifying an increase in the serum and CSF Sn-IgM concentrations in experimentally infected horses is indicative of an acute infection with S. neurona, and may lead to an additional diagnostic tool for EPM disease detection.
3.6. Literature Cited


Figure 3.1: Western blot results comparing pre-inoculation samples (Group 1A) (Lanes 1-6), post-inoculation samples (Lanes 1-6), negative control (Lane 7) and positive control (Lane 8).
Figure 3.2: Groups 1A and 1B average PP values from week 0 to week 7. Weeks 0 - 4 had 12 horses (Groups 1A and 1B) at each time point; weeks 5 - 7 had 6 horses (Group 1B) at each time point. (Error bars represent two standard errors.)
Figure 3.3: Groups 2B, 2C, 2D and 2E PP values from week 0 to week 4. (Error bars represent two standard errors.)
Figure 3.4: Box and whisker plots of PP values for pre-inoculation CSF of Group 1A; post-inoculation CSF of Group 1A; pre-inoculation CSF of Group 1B; and post-inoculation CSF of Group 1B.
Figure 3.5: Box and whisker plots of OD values for negative and positive control horses and WNV positive horses.
Figure 3.6: Correlation between neurologic score and PP. ($R^2$ of 0.2932)
LITERATURE CITED

Chapter 1


62


Chapter 2


68


71


Chapter 3


