Equine Protozoal Myeloencephalitis: Investigating Immunopathogenesis and Treatment Efficacy in Mouse Models and Clinically Affected Horses

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Sciences

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December 12th, 2019
Blacksburg, VA

Keywords: Equine Protozoal Myeloencephalitis, interferon gamma, EPM treatment

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ABSTRACT

Equine protozoal myeloencephalitis (EPM), predominantly caused by the protozoa Saracocystis neurona, is a common neurologic disease in horses from North America. Equine exposure to the parasite occurs frequently as the protozoa is excreted in opossum (Didelphis virginiana) feces and contaminates the horse’s environment. However, clinical neurologic disease only emerges in a small fraction of exposed horses. The seemingly protective immune response that develops in some exposed horses but not all is not fully defined. Previous reports utilizing horse EPM models and immune compromised mouse models, which develop disease simulating EPM after infection with S. neurona, have reported a role of T-lymphocytes and the cytokine interferon gamma, in disease protection. As part of this dissertation, the role of T-lymphocytes and IFNγ was further elucidated. It was determined that IFNγ production is essential for T-lymphocytes to offer protection against S. neurona induced encephalitis, in immune compromised mice. Another factor hindering prognosis of EPM affected horses is treatment failure. The efficacy of the antiprotozoal decoquinate, was tested and found to be ineffective at preventing S. neurona encephalitis, in immune compromised mice. However, the antiprotozoal, diclazuril, was found to be effective at preventing S. neurona encephalitis in immunocompromised mice but once treatment was terminated, infection persisted, and neurologic disease developed. In-situ methods were employed to extensively evaluate the immunopathology of spinal cord tissue samples collected from EPM affected horses. A novel in-situ hybridization technique was successfully utilized to identify S. neurona in
tissue samples collected from horses with EPM. This technique will create new opportunities for investigating the immunopathology of EPM. Overall results from the studies conducted in this dissertation suggest that IFNγ production from T lymphocytes is essential for them to offer protection against *S. neurona* encephalitis. Additionally, further insight on FDA approved and non-FDA approved treatment options for *S. neurona* infection was gained through the use of the B6Ifnγ-/- mouse model. Collectively, these studies expanded on the knowledge of an understudied equine neurologic disease.
General Public Abstract

Horses are susceptible to the neurologic disease Equine Protozoal Myeloencephalitis, more commonly referred to as EPM by equine enthusiasts. The disease results from ingestion of the parasite, Saracocystis neurona, which contaminates the horse’s natural environment; therefore, horses are likely to come in contact with the parasite while eating or drinking. Not all horses that encounter S. neurona develop neurologic disease, some will be protected by their immune system with the only evidence of exposure being serological antibodies. In efforts to not experimentally induce EPM in horses, an immunocompromised mouse model is often used instead. Through the use of the immunocompromised mouse model, researchers have discovered that the immune cell, T lymphocytes, and signaling molecule, interferon gamma, are important for protection against S. neurona infection. In one study conducted for this dissertation it was found that T lymphocytes need to be able to produce interferon gamma in order to provide protection. Another issue that the immunocompromised mouse model has helped address, is EPM treatment efficacy. The inability of antiprotozoal drugs that are utilized for EPM treatment to fully eliminate the parasite from the horse’s body is thought to cause reoccurring disease in some horses. One non-FDA approved treatment was evaluated here and determined not to be effective in the immunocompromised mouse model. One FDA approved treatment option, which is commonly used to treat EPM, was evaluated as well. This drug was proven to be effective at preventing disease while mice were being treated but termination of treatment led to development of neurologic disease, exemplifying treatment failure. One final study was conducted to examine the different types of immune cells and signaling molecules in spinal cord tissue samples collected,
from horses which had to be euthanized due to poor prognosis related to EPM. In this study a novel experimental technique was successfully used which will help progress EPM research. Overall results of these studies offered more explanation on the immune response that protects against neurologic disease from *S. neurona* infection and demonstrated that not all treatments are effective and reoccurring disease may be a result of treatment failure.
Acknowledgements

The research performed in this dissertation could not have been accomplished without the support of my mentor Caroline Leeth and faithful lab mate Jing Zhu. I’d also like to acknowledge all the contributions made by undergraduate laboratory member Ashley Potter and previous Leeth lab member Leah Kasmark. My committee helped me greatly throughout my graduate student career. My graduate student accomplishments would have been so much more difficult to achieve without the support of my husband and family.
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Attribution

The co-authors Jing Zhu, Leah Kasmark, David Lindsay and Caroline Leeth contributed to the experimental work involved in the study in Chapter II. Caroline Leeth also contributed knowledge and guidance for the study in Chapter II.

Tanya LeRoith is a board-certified pathologist and reviewed cerebellum slides for encephalitis in the published study conducted in Chapter II.

Sharon Witonsky contributed knowledge and guidance for the study conducted in Chapter II.

The manuscript in Chapter II is published and permission for use in this dissertation was granted.

Dr. Siobhan Ellison contributed one of the medications tested in Chapter III.
Chapter I: Introduction

Reports of horses developing idiopathic neurological disease began in the late 1960s (Pricket et al, 1968 and Rooney et al, 1970) and postmortem examination revealed protozoa and inflammatory lesions. The protozoan was originally expected to be *Toxoplasma gondii* and later determined to be another *apicomplexan* species, *Sarcocystis* sp., thus the neurologic disease became known as equine protozoal myeloencephalitis (EPM) (Beech, 1974 and Mayhew, 1977). Over a decade had elapsed before the etiological protozoa was isolated from an EPM affected horse and continuously propagated in culture (Dubey, 1991). It was officially identified as a member of the *Sarcocystis* species and was suitably named *Sarcocystis neurona*, given the frequent finding of the protozoa in the central nervous system (CNS) of the affected host (Dubey, 1991). In subsequent studies *S. neurona* was successfully isolated from CNS tissue collected from EPM affected horses (Davis et al, 1991, Bowman et al, 1992). Disease simulating EPM occurred in horses or immunodeficient mice after infection with *S. neurona*, which provided proof of concept that protozoal myeloencephalitis arose from *S. neurona* infection. The opossum was identified as the definitive host of *S. neurona* (Dubey and Lindsay, 1998), excreting *S. neurona* sporocysts in its feces, contaminating the horse’s environment and exposing them to ingestion of *S. neurona*. The intermediate host include, raccoons (Stanek et al, 1998 and Dubey, 2001), armadillos (Cheadle et al, 2001a), cats (Dubey, 2000) and skunks (Cheadle et al, 2001b), and harbor sarcocysts in muscle tissue. The horse is a dead-end host as only asexual protozoal life stages, schizonts and merozoites, and no sarcocysts or sporocysts are found in the horse (Dubey et al, 1991, Davis et al, 1991, Bowman et al, 1992).
Approximately 1% of horses which encounter *S. neurona* will develop neurologic disease but the seroprevalence of *S. neurona* antibodies ranges from 15-89% of tested horses (Reed et al, 2016). The seroprevalence variance is influenced by geographic region and presences of sera antibodies merely indicates exposure to *S. neurona* and not clinical neurologic disease (Reed et al, 2016). The high exposure rate but relatively low incidence of neurologic disease, most likely indicates that most exposed horses elicit the required immune response to prevent clinical neurologic disease. The immunopathogenesis of EPM is not completely understood and a better understanding would improve diagnostic tests and treatment options of EPM. The current EPM immunopathogenesis knowledge has mostly been acquired by employing immunocompromised mouse models.

Marsh et al (1997) was of the first investigators to report that immune compromised mice lacking T lymphocytes (C57Bl/6 Foxn1nu), succumbed to EPM like disease after infection with *S. neurona* merozoites. Following this investigation Dubey and Lindsay (1998) discovered robust *S. neurona* protozoal encephalitis in Balb/c interferon gamma (Ifγ-/−) mice, initiating the common use of this mouse as a model for *S. neurona* infection and neurologic disease. Both the Balb/c and C57Bl/6 mouse strains with the *Ifγ* gene knockout develop neurologic abnormalities on average within 30 days post infection, and commonly serve as positive control mice in investigations employing other immune compromised mice (Rosypal et al, 2002, Witonsky et al, 2003, Witonsky et al, 2005a, Witonsky et al, 2005b, Hay et al, 2019). The B6.129P2- β2m tm1/unc knockout mouse, void of MHC I expression and consequently lacking IFNγ producing CD8 + T lymphocytes, developed encephalitis as a result of *S. neurona* infection (Witonsky et al,
The B6 Ifnγ -/- mouse is most commonly utilized for investigating EPM and was used in the current dissertation for multiple studies.

The B6 Ifnγ -/- mouse was used in this dissertation to investigate the potential of treatment failure being a result of disease relapse which occurs in some EPM affected horses (MacKay et al, 2006, Hay et al, 2019). This mouse model was also used in this dissertation to test the efficacy of antiprotozoal drug decoquinate against S. neurona infection in the B6 Ifnγ -/- mouse. Additional study objectives of this dissertation included the use immunocompromised mouse models which had not previously been infected with S. neurona to further investigate the immunopathogenesis of EPM - like disease in the mouse. This dissertation also included a study which explored different in-situ methods to investigate pathological changes in spinal cord tissue samples collected from clinically EPM affected horses and neurologically normal horses. The overall goal of this dissertation was to gain further knowledge on immunopathogenesis and treatment options to improve prognosis for horses affected with clinical EPM.

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Chapter II: Review of Literature

Introduction

Reports of horses developing idiopathic neurological disease began in the late 1960s (Pricket, 1968 and Rooney, 1970) and postmortem examination revealed protozoa and inflammatory lesions. The protozoan was originally expected to be Toxoplasma gondii and later determined to be another apicomplexan species, Sarcocystis sp., thus the neurologic disease became known as equine protozoal myeloencephalitis (EPM) (Beech, 1974 and Mayhew, 1977). Over a decade had elapsed before the etiological protozoa was isolated from an EPM affected horse and continuously propagated in culture (Dubey, 1991). It was officially identified as a member of the Sarcocystis species and was suitably named Saracocystis neurona, given the protozoa’s preference for replication in the central nervous system (CNS) of the affected host (Dubey, 1991). In subsequent studies S. neurona was successfully isolated from CNS tissue collected from EPM affected horses (Davis et al, 1991, Bowman et al, 1992). Disease simulating EPM occurred in horses or immunodeficient mice after infection with S. neurona, which provided proof of concept that protozoal myeloencephalitis arose from S. neurona infection. The opossum was identified as the definitive host of S. neurona (Dubey and Lindsay, 1998), excreting sporocysts in its feces, contaminating the horse’s environment and exposing them to ingestion of S. neurona. The transmission of S. neurona sarcocysts, which are found in the muscle of a slew of intermediate hosts, including racoons (Stanek et al, 1998 and Dubey, 2001), armadillos (Cheadle et al, 2001a), cats (Dubey, 2000) and skunks (Cheadle et al, 2001b), results in the production of sporocysts in the opossums intestines indicating that the opossum is the definitive host of S. neurona. The horse is a dead-end
host as only asexual protozoal life stages, schizonts and merozoites, and no sarcocysts or sporocysts are found (Dubey et al, 1991, Davis et al, 1991, Bowman et al, 1992). Molecular characterization has differentiated *S. neurona* from other sarcocystis species found in opossums including *S. falcatus*, *S. lindsayi* and *S. speeri* (Marsh et al, 1999, Tanhauser et al, 1999, Rosenthal et al, 2001). After identification of *S. neurona* as the etiological agent of EPM, it was determined that infection with *Neospora hughesi* also result in neurologic disease in susceptible horses (Marsh et al, 1996). However, the predominant cause of EPM remains as *S. neurona*, as occurrences of *N. hughesi* histologically is rare and prevalence of *N. hughesi* antibodies in EPM affected horses is less common than *S. neurona* antibodies (James et al, 2017). The seroprevalence of *S. neurona* antibodies ranges from 15-89% of horses depending on geographic region and presences of sera antibodies merely indicates exposure to *S. neurona* and not clinical neurologic disease (Reed et al, 2016). There seems to be no conclusive risk factors with reports of disease occurring in various breeds and age of horses, immunosuppression related to excessive transport or performance may increase risk (Cohen et al, 2007 and Morley et al, 2008). The high exposure rate but relatively low incidence of neurologic disease, most likely indicates that most exposed horses elicit the required immune response to prevent clinical neurologic disease. The immunopathogenesis of EPM is not completely understood and a better understanding would improve diagnostic tests and treatment options of EPM. The current knowledge of diagnostic tests, treatments and immunopathogenesis of *S. neurona* in both the horse and experimental mouse models is covered in this review.

**Antemortem, post-mortem and differential diagnosis of EPM**
**Diagnostic assays**

Antemortem diagnosis of EPM is dependent on the presences of at least serum *S. neurona* antibodies, response to anti/protozoal treatment and clinical neurologic signs which vary from muscle atrophy, ataxia, proprioception loss to more severe signs such as seizures, depending on the location of the pathological lesion (Dubey et al, 2015). Initially positively identifying *S. neurona* antibodies was limited by available assays but knowledge of parasite biology improved and expanded on detection assays. This helped to positively identify EPM affected horses and advance the knowledge of EPM.

Originally western blot analysis, which reportedly demonstrated no cross reactivity with other *Saraocystis* species including, *S. cruzi, S. fayeri* and *S. muris*, was utilized for detection of sera and CSF antibodies against *S. neurona* (Granstrom et al, 1993). The specificity of the western blot turned out to be less than ideal and was improved by Rosano et al (2000), by introducing the use of bovine serum containing *S. cruzi* antibodies as part of the blocking solution for the western blot. Direct agglutination tests were developed in the form of sera agglutination test which was successfully utilized experimentally by Lindsay and Dubey (2001) and indirect fluorescent antibody test (IFAT) was utilized as a clinical diagnostic tool, demonstrating greater sensitivity and specificity than the western blot (Durate et al, 2003). After generation of recombinant *S. neurona* surface antigen (SAG) protein-1, SnSAG-1 (Ellison et al, 2002), an enzyme linked immunosorbent assay (ELISA) was generated for the specific detection of antibodies against the SnSAG-1 (Ellison et al, 2003). Commercially available diagnostic assays for *S. neurona* antibody testing include; western blot, IFAT and various SAG ELISAs (Reed et al, 2016).
Surface antigens are present on other apicomplexan parasites such as *Toxoplasma gondii*, and have presumably conserved function, relating to host cell adhesion and invasion, contributing to the parasite’s virulence (Lekutis et al, 2001). The presences of SAGs were originally suggested by Liang, et al (1998) when two proteins, Sn14 and Sn16, were identified by western blot. Neutralization of these proteins by antibodies from sera and CSF collected from EPM affected horses, impeded replication of *S. neurona* schizonts *in vitro*. In addition to SnSAG-1, Howe et al (2005) named three other SnSAGs referred to as SnSAG 2,3 and 4. Recognition of SnSAGs 2,3 and 4, expanded the ELISAs available for EPM diagnostics and greatest sensitivity and specificity was noted with the SnSAG 2 ELISA and the least sensitive being SnSAG-1 ELISA (Hoane et al, 2005). Discovery that not all *S. neurona* isolates expressed SnSAG-1 but frequently expressed SnSAG 2,3 and 4 (Howe et al, 2008), explained the lack of sensitivity of the SnSAG-1 ELISA observed by Hoane et al (2005). *Saracocystis neurona* isolates lacking SnSAG-1 expressed a parologue referred to as either SnSAG 5 or SnSAG6 (Crowdus et al, 2008 and Wendte et al, 2010). The discovered variation in surface antigen expression amongst different *S. neurona* isolates meant diagnostics needed to be further improved, which led to the development of polyvalent ELISA. The engineered ELISA with chimeric recombinant protein SnSAG 4/3 utilized along with SnSAG 2 ELISA was identified as the optimal ELISA assays for *S. neurona* antibody detection in sera and CSF samples (Yeargan et al, 2011). To improve testing efficiency without sacrificing accuracy, a trivalent ELISA was later engineered to detect antibodies against SnSAG 2,3 and 4 (Yeargan et al, 2015). Reed et al (2013), found that a serum: CSF antibody ratio provides the most accurate antemortem diagnostic test for EPM, as it accounts for passive
transfer of sera antibodies across the blood brain barrier (BBB), decreasing false positives related to CSF antibody detection. With the SAG 2,3,4 ELISA, the serum: CSF ratio indicative of EPM is ≤ 100 (Reed et al, 2013). Optimal antemortem diagnostic assays are essential for any non-terminal immunopathogenesis studies in horses naturally affected by EPM and additional biomarkers for confirming EPM and differentiating it from other neurologic diseases would help progress the understanding of EPM.

**Differential Diagnosis**

Due to the assortment of neurologic signs that can present in EPM affected horses, EPM can be mistaken for other neurologic diseases or lameness. Other neurologic diseases affecting the horse include infectious diseases such as; equine herpes myeloencephalopathy, west nile viral encephalomyelitis, eastern equine encephalomyelitis and rabies. The mentioned infectious diseases often occur in unvaccinated horses, especially rabies, and most are accompanied by fever, whereas EPM affected horses rarely develop a fever. Non-infectious diseases of the spinal cord such as cervical vertebral stenotic myelopathy (CVSM) primarily manifests as neurologic abnormalities in the hind limbs and radiographs often reveal vertebrae column abnormalities, which cause compression on the spinal column (Zacchary et al, 2012). Another common neurologic disease in horses is neuroaxonal dystrophy which progresses to equine degenerative myeloencephalopathy (EDM) and is a result of chronic degeneration of the cell body and axons of neurons. This degeneration hinders neuronal signaling, generally causing hypermetric gait and symmetric ataxia (Finno, 2011). There are no definitive diagnostic assays for NAD/EDM, but improvement can be seen in some
horses supplemented with Vitamin E. Typically NAD/EDM is observed in horses 4 years of age or younger and the disease is thought to be heritable (Finno, 2011). Since it is not currently possible to definitely diagnosis EPM (antemortem), it’s hard to determine if neurologic horses which do not have a S. neurona serum:CSF antibody ratio indicative of EPM and/or do not respond to appropriate treatment, are afflicted by another neurologic disease such as EDM, or simply just may not fall within the typical criteria used for EPM diagnosis. Therefore, in efforts to differentiate EPM from other neurologic diseases, further diagnostic studies investigating other potential biomarkers such as phosphorylated neurofilament H (pNF-H), C reactive protein and serum amyloid A have been conducted.

The acute phase proteins, C-reactive protein and serum amyloid A were not reported to be elevated in a small study examining this protein concentrations in serum and CSF of equids with EPM or CVSM (Mittelamn et al, 2018). However, it was confirmed that pNF-H can be detected in both the serum and CSF of horses and horses with EPM and had greater serum concentrations of pNF-H than horses suffering from CVSM (Intan-Shameha et al, 2017). The neurofilament (pNF-H) accumulates in the serum and CSF of other mammalian species as a result of neuronal and axonal degeneration, which can result from neuroinflammation (Lyman et al, 2014) and neuroinflammation pathologically in EPM horses. Reports of elevated serum pNF-H concentrations in horses with EMD have been reported (Gomez et al, 2019) but how levels differed from EPM affected horses was not described. The use of pNF-H as a biomarker for EPM deserves further investigation and it may also help explore disease progression or status in horses with chronic or relapsing EPM. Any additional biomarkers that could be utilized to confirm EPM diagnosis would help improve prognosis of
affected horses by offering further explanation on disease pathogenesis which could help broaden treatment options and supportive therapies.

**Post Mortem Diagnosis**

Currently the only definitive means of diagnosing EPM is findings of pathological lesions consistent with EPM. Histologic changes associated with EPM consist of focal or multi focal areas of immune and neural cell infiltrates, which typically include a combination of lymphocytes, neutrophils, eosinophils, multinucleated giant cells, gitter cells, foci of perivascular cuffing and gliosis, and necrotic tissue (Dubey et al, 2001b). In order to pathologically characterize EPM lesions, *S. neurona* needed to be identified within the lesion site (Boy et al, 1990, Dubey et al, 1974, Granstrom et al, 1992). It proves difficult to find within the equine CNS even when characteristic lesions are identified and *S. neurona* antibody titers correlate with disease. When *S. neurona* merozoites or schizonts are identified in the CNS they are most commonly found in the cytoplasm of neurons and monocytes. The addition of immunohistochemistry staining for *S. neurona* in collected tissue samples significantly enhances chances of finding *S. neurona* (Dubey et al, 1999, Dubey and Hamir, 2000). Multi focal or focal lesions can appear throughout the spinal cord and at times are found in the brain, particularly the brainstem (Dubey et al, 2015). Given the inflammatory nature of EPM lesions within the CNS, it is reasonable to hypothesize that the immune response against *S. neurona* within the CNS is majorly responsible for the CNS damage and subsequent neurologic signs. Whether the infrequent finding of *S. neurona* stages in horses with EPM, even in horses seemingly acutely affected prior to euthanasia and necropsy, is due to lack of parasite
presences or the difficulty of locating a microscopic parasite in a larger CNS, remains unknown.

**Treatment options**

*Sulfadiazine/pyrimethamine*

Historically, treatment plans for EPM affected horses consisted of sulfonamide (sulfadiazine) and pyrimethamine drugs which are traditionally used to treat other Apicomplexan infections. The drugs have a synergistic effect which causes a disruption of tetrahydrofolate synthesis which in turn interrupts nucleic acid synthesis affecting the replication of *S. neurona* merozoites (Lindsay and Dubey, 1999). The sulfadiazine/pyrimethamine mixture is commercially labeled as ReBalance (PRN, Pharmacal) and is an FDA approved treatment. The recommended treatment duration for EPM with ReBalance is 90 days which is longer than other treatment options due to the short half-life of the drug which influences CNS concentrations. This prolonged treatment period can lead to complications such as gastrointestinal upset and reproductive complications (Pusterla and Tobin, 2017). While relapse of neurologic deficits after treatment cessation can be an issue related to all EPM treatments the greatest percentage of relapse (25%) seems to be associated with sulfadiazine/ pyrimethamine treatment (Fenger et al, 1998). The usage of sulfadiazine/pyrimethamine treatment for EPM is declining and more frequently treatment plans encompass the Benzeneacetonitrile agents: diclazuril and toltrazuril sulfone.

**Ponazuril and Diclazuril**

In both diclazuril and toltrazuril sulfone (ponazuril) the mechanism of action against *S. neurona* is related to direct action on the apicoplast, a chloroplast like structure
present in *S. neurona* but not in the horse (or other mammals). Both diclazuril (Dirikolu et al, 1999) and ponazuril (Furr and Kennedy, 2001) have more favorable pharmacokinetics than sulfadiazine/pyrimethamine and therapeutic CNS concentrations are easily maintained with a recommended standard treatment plan of 28 days. In addition to being, two FDA approved treatment options for EPM, both drugs especially diclazuril, have decreased relapse rates and demonstrate promising prophylactic effects.

The activity of ponazuril against *S. neurona* merozoite replication was proven in culture (Lindsay et al, 2000) and its efficacy as a treatment for clinically affected EPM horses was investigated and established (Furr, 2001). Furr et al (2001) found that ponazuril (10mg/kg) successfully improved neurologic deficits by one neurologic grade or more and/or horses became negative for CSF *S. neurona* antibodies after treatment. When a single dose of ponazuril is given to mice at 4-14 days post infection (DPI) with *S. neurona*, the onset of neurologic signs is delayed and the number of parasite in the brain is decreased, suggesting that even a single dose of ponazuril improved prognosis in the immune compromised state (Franklin et al, 2003). Likewise, weekly administration of ponazuril in experimentally infected horses greatly reduced seroconversion and clinical signs suggesting weekly doses of ponazuril may have preventive effects (MacKay et al, 2008). Additionally, in experimentally *S. neurona* infected young horses, the administration of ponazuril at a dose of 2.5 mg/kg or 5 mg/kg for one week prior to infection and throughout the study period, decreased the occurrence of neurologic signs, 71 or 40%, respectively. This was a significant improvement from infected untreated horses which all developed clinical disease (Furr et al, 2006). The reported relapse rate
within 4 months post treatment with ponazuril in naturally affected horses, treated daily for 28 days, was 8% (Furr et al, 2001).

The suspected relapse rate of diclazuril in naturally affected EPM horses is estimated to be 5% (Bentz et al, 2000) with relapse being defined by the recurrence of neurologic signs within 6 months of terminating treatment, and no experimental horse model studies have been conducted to investigate relapse after diclazuril treatment. However, mouse model studies demonstrate that diclazuril effectively inhibits *S. neurona* replication in treated infected immune compromised mice (Dubey et al, 2001c) but within 60 days of cessation of treatment mice develop neurologic disease, exemplifying the cooperation of treatment and the host’s immune system for eliminating the infection (Hay et al, 2019). This is supported by *in-vitro* work which diclazuril inhibited the activity of *S. neurona* merozoites and limited replication but did not eliminate it (Lindsay and Dubey, 2000). Additionally, reports of prophylactic effects of diclazuril are demonstrated in foals treated with a low dose of diclazuril had significantly less sera *S. neurona* antibodies compared to untreated foals, despite residing in an EPM endemic area (Pusterla et al, 2015). Likewise, a recent pharmacokinetic study has determined that a low dose of diclazuril administrated every 3-4 days achieves plasma concentrations which are known to inhibit *S. neurona* replication, highlighting the use of diclazuril for prophylactic treatment in EPM endemic areas or horses with reoccurring disease (Hunyadi et al, 2018). Additional pharmacokinetic studies had previously been conducted evaluating the suggested treatment dose (1mg/kg) compared to the prophylactic dose of 0.5mg/kg (Hunyadi et al, 2015). The previously discussed medications,
sulfadiazine/pyrimethamine, diclazuril and ponazuril, are the only FDA approved EPM
treatment options, the efficacy of other drugs has been investigated as well.

**Pyrantel Tartrate**

The anthelminthic drug, pyrantel tartrate, demonstrated activity against *S.
neurona* merozoites in culture (Kruttlin et al, 2001) but when prophylactically given to
interferon gamma knockout mice no activity against *S. neurona* sporocyst infection was
observed (Lindsay and Dubey, 2001). In an experimental horse model study, which dosed
weanling foals with a low dose of *S. neurona* sporocysts daily for 118 days, to
supposedly mimic natural exposure, no treatment effect was found. In the pyrantel study,
most horses (10 of 12) in each group seroconverted regardless of treatment status and
only one horse displayed clinical neurologic signs (Rossano et al, 2005a).

**Decoquinate**

The quinolone anticoccidial drug, decoquinate, compounded with
immunomodulator, levamisole, has been investigated as therapeutic treatment option as
well. In a clinical field trial horses, which were presumably diagnosed with EPM based
on clinical neurologic signs and positive sera titers, were treated with the decoquinate/
levamisole compounded medication and improvement in neurologic abnormalities were
observed (Ellison et al, 2012). Lindsay et al (2013) demonstrated that *in-vitro*
decoquinate inhibited merozoite replication of two isolates of *S. neurona* merozoites, one
opossum (SnOP-15) and one equine (Sn6) derived, and at high enough concentrations
eliminated developing schizonts. While these two studies promisingly evaluate the
potential of decoquinate as EPM therapeutic, additional studies investigating the
pharmacokinetics of the drug and efficacy in the horse without levamisole should be conducted.

**Bump Kinase Inhibitors**

A class of drugs known as bump kinases inhibitors, which work against the calcium dependent protein kinase -1 in *S. neurona*, have also been investigated as a treatment option (Ojo et al, 2016). Specifically, the bump kinase inhibitor-1553, prevented neurologic disease in *S. neurona* infected gamma interferon knockout mice, while all infected untreated mice developed disease. No histological signs of *S. neurona* or lesions were observed in the brains of treated mice and only 10% of mice developed sera antibodies against *S. neurona* (Ojo et al, 2016). This study introduced a novel class of drugs into the repertoire of potential EPM treatment options and should be further investigated in the horse.

**Conclusions**

While current treatment plans employing diclazuril or ponazuril drastically improve prognosis for affected horses there still are a number of horses, which relapse within six months of treatment cessation most likely due to treatment failure and therefore additional treatment strategies need to continue to be investigated. A high-throughput drug screen identified 18 drugs which disrupted *S. neurona* growth such as the compound, dantrolene (Bowden et al, 2018). Novel medications for treatment of EPM should continue to be explored, as well as further elucidating the immunopathogenesis of EPM in order to target immunomodulators to support current treatments which seem to be most effective with the assistance of the host’s immune system. Several models are currently used to investigating the protective immune response against *S. neurona*,

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experimental mouse models, experimental infection in horses and use of samples collected from naturally affected horses.

**The experimental EPM model: Horse**

One of the first attempts to experimentally reproduce EPM was conducted by Fenger et al (1997), when *Sarcocystis* sporocysts were orally inoculated into seven *S. neurona* naïve foals, 3 of which received repeated (2-3 doses) inoculation of 1-to 10 million sporocyst over a time span of 42 days. Two foals received single doses of 20-40 million sporocysts and two additional foals served as controls and did not receive sporocysts. While all foals developed neurologic deficits, sera and CSF antibodies against *S. neurona* and histological inflammatory lesions were observed in the CNS of 3 of 5 infected foals, no parasite was detected or retrieved in cultured CNS tissue samples. These results help support the hypothesis the opossum was the definitive hose of *S. neurona* (Fenger, 1997).

Cutler et al (2001) infected naïve Canadian yearling horses which were either subjected to immunosuppression by dexamethasone or not, with *S. neurona* sporocyst collected from wild opossums. The horses were given $5 \times 10^5$ sporocysts daily for seven days which resulted in an immune response against *S. neurona* as measured by serum and CSF antibodies in all horses. The horses treated with dexamethasone throughout developed other infections such as pneumonia which complicated interpretation of the study results. The neurologic signs exhibited by foals in this study were mild and, in some cases, improved throughout the course of the study. The investigators were not able to find *S. neurona* in collected CNS tissue samples, blood or CSF in any of the infected horses. Although EPM like disease was observed in the horses of this study it was not compatible in severity to clinical EPM despite the repetitive dosing and immunosuppression (Cutler
et al, 2001). Lindsay et al (2000) inoculated culture derived *S. neurona* merozoites, which were originally harvested from an EPM affected horse, directly into the CNS. This attempt to induce clinical EPM did not result in *S. neurona* induced encephalitis; all horses demonstrated CSF antibodies to *S. neurona* which waned with time and sera antibodies were produced as well, but neurologic abnormalities were not observed nor were histological lesions. Proceeding investigations attempted to induce immunosuppression in horses with corticosteroids or lengthy transport, in order to induce more severe *S. neurona* infection in efforts to recover the parasite from the CNS. Saville et al (2001) subjected 12 Canadian *S. neurona* naïve foals to a lengthy transportation session and inoculated foals directly after arrival to the housing facility or 14 days after arrival of which some foals were treated with dexamethasone in hopes of achieving further immune suppression. The foals in this study were orally infected with sporocysts harvested from intestines of opossums and the exact number of viable *S. neurona* sporocysts was unknown. The inoculum utilized in the study was also given to interferon gamma knockout mice, which demonstrated neurologic disease as a result of *S. neurona* induced encephalitis, proving pathogenicity of the inoculum. All foals demonstrated antibodies against *S. neurona* in circulation (sera) and in CSF and mild neurologic deficits were observed. The neurologic deficits were not compatible to what is typically observed in naturally affected horses and some affected horses were demonstrating neurologic improvement by the end of study without treatment. Necropsy of all foals revealed inflammatory lesions but *S. neurona* was not observed in the lesion sites, the most severe lesions and clinical signs were observed in the foals that did not receive dexamethasone, suggesting that the dexamethasone hampered the inflammatory response
improving pathology (Saville et al, 2001). Once the life cycle of *S. neurona* was fully elucidated (Dubey, 2000) and confirmed, the usage of laboratory raised racoons and opossums (Stanek et al, 2002) to harvest known quantities of *S. neurona* sporocysts opened new opportunities for establishing a horse model which recapitulated clinical EPM.

Utilizing a two-phase transport induced immunosuppression method, Saville et al (2002) infected *S. neurona* naïve foals with one dose of $1.5 \times 10^6$ *S. neurona* sporocyst from laboratory opossums. All infected foals developed moderate neurologic signs 5-7 days post infection (DPI) and inflammatory lesions were found at necropsy in some foals but *S. neurona* was not seen. The results of the study were not altered by the second transport as there were no notable differences between single transport and double transport groups. Another study utilizing Canadian foals subjected to transport induced stress were inoculated with doses of *S. neurona* sporocysts which varied from $10^2$ to $10^6$ collected from laboratory raised opossums. In general, as the dose of *S. neurona* sporocyst increased, the occurrence of sera and CSF antibodies against *S. neurona* increased, and incidence and severity of neurologic signs also increased and the largest dose of sporocysts resulted most consistently in histological inflammatory lesions but no *S. neurona* was found. The changes observed between each group were not consistent amongst all horses (n= 4/group) in the group suggesting differences in individual immune response (Sofaly et al, 2002). Elitsur et al, (2007) attempted to map out the migration pattern of *S. neurona* in horses by infecting six ponies with one large dose of sporocysts ($250 \times 10^6$ ) from the laboratory Racoon-Opossum and one pony was necropsied 1,2,3,5,7, and 9 DPI. Initially, *S. neurona* schizonts were found in the mesenteric lymph
node followed by the liver, then the lung and by 7 and 9 DPI inflammatory lesions were noted in the CNS of necropsied ponies but no parasite was found. The ponies in this study remained clinically normal and whether neurologic deficits would have developed over time or the immune system would have resolved the infection within the CNS is unknown.

In most of the previously discussed studies young horses of weanling or yearling age were utilized as study subjects which in most cases created issues relating to respiratory ailments or exacerbated helminth infections due to immune suppression and the young and immature immune system of the young horse (Perkins and Wagner, 2015). While young horses provided naïve subjects for developing the EPM model, it added in additional complications that often skewed study results. Likewise, it is unknown whether horses that present with clinical EPM were previously exposed or not, given the high seroprevalence it is likely that horses with clinical EPM were previously exposed to *S. neurona*.

While the previously discussed attempts to create an EPM- horse model did successfully induce neurologic disease, results were inconsistent and required immune suppression making the models not ideal for investigation of immunopathogenesis and vaccine studies. Proceeding these attempts to experimentally induce clinical EPM, it was investigated whether intravenous inoculation of autologous leukocytes which had been cultured with *S. neurona* merozoites, would result in more robust neurologic signs that more closely paralleled clinical EPM (Ellison et al, 2004). This methodology did result in the onset of neurologic signs more consistent with clinically affected horses and *S. neurona* was recovered from the CNS tissue samples of infected horses (n=4) (Ellison et
al, 2004). Although this model does not mimic natural oral exposure to *S. neurona* in horses and intravenous inoculation bypasses the barrier of the gastrointestinal tract, it did offer reproducible infection without immunosuppression which hinders the ability to study immunopathogenesis.

**Immunopathogenesis in the horse model**

The reintroduction of autologous lymphocytes infected with *S. neurona* merozoites model (Ellison et al, 2004) was utilized by Witonsky et al (2008) to investigate immune responses in infected horses. All horses in the study developed moderate neurologic signs by 15 DPI and were sera and CSF positive for SnSAG1, *S. neurona* antibodies by the end of the study at 55 DPI, indicating an immune response as these horses did not have *S. neurona* CSF antibodies prior to infection. Furthermore, at various time points throughout the study investigators found that peripheral blood leukocytes (PBLs) collected from the *S. neurona* infected horses had increased antigen specific CD8+ T lymphocyte responses after 24 hour in-vitro stimulation with *S. neurona* merozoites compared to control horses. The increase in antigen specific response was determined by increased number of CD8+ T lymphocytes and increased interferon gamma production. Additionally, PBL collected from *S. neurona* infected horses demonstrated decreased cellular proliferation after in vitro stimulation with PMA/I compared to control horses. The decreased proliferative immune response to PMA/I was also observed in PBLs collected from horses with clinical EPM, from natural infection which was defined by *S. neurona* antibodies in the CSF and neurologic signs (Yang et al, 2006). Yang et al (2006) found significant increase in CD4+ T lymphocytes in circulation in the EPM affected horses compared to the control horses which were serologically positive for *S.
neurona antibodies but neurologically normal. Lewis et al (2014) utilized the EPM induction model described by Ellison et al (2004) and found that infected horses progressed in severity of neurologic disease over the course of the 70-day study period. There were some immune response changes relating to S. neurona infection such as apoptotic changes, increased PBL proliferative response to PMA/I which was opposite of what was previously observed (Yang et al, 2006 and Witonsky et al, 2008) and a decreased proliferative response to S. neurona in vitro stimulation (Lewis et al, 2014).

The method described by Ellison et al (2004), does consistently result in moderately severe neurologic signs and collectively the discussed results of studies utilizing this model, suggest an altered antigen specific response in S. neurona infected horses. Other researchers have utilized tissue samples collected from naturally affected EPM horses to evaluate immunopathology.

The finding of S. neurona in the CNS tissue during postmortem examination can be difficult but this allows for in situ examination which has provided opportunities for investigation of the immune response within the CNS in EPM affected horses. Scott et al (2005) evaluated lymphocyte population in EPM identified lesion sites, in formalin fixed paraffin embedded CNS tissue samples collected from EPM affected horses (n=17). The examined sections were replicates of those which S. neurona was found. In the analyzed sections there were significantly greater percentages of lymphocytes in the samples from EPM affected horses compared to control horses, consisting primarily of CD3+ T lymphocytes. Although S. neurona could not be identified directly in the examined sections due to limited experimental capabilities, results of this in-situ studied are insightful.
Investigations comparing immune cells and immune mediators circulating in the CSF and blood of EPM affected horses to neurologically normal healthy horses have also provided insight into the immune response which is thought to be protective against *S. neurona* myeloencephalitis. In a small study examining the different T-lymphocyte subsets in the CSF of EPM affected horses (n=4) and healthy horses (n=7) found a significant increase in CD8+ T-lymphocytes in EPM horses (Furr, 2001). While the small population size in this study is potentially skewing the findings, they are supported by other studies which noted differences in cell mediated immune response influenced by *S. neurona* infection (Scott et al, 2005, Witonsky et al, 2003, 2008, Yang et al, 2006). A brief study examining the gene expression profile of several cytokines, including IFNγ, found that lymphocytes isolated from clinically affected EPM horses and stimulated *in-vitro* with SnSAG1 protein, had a delayed expression of IFNγ gene expression compared to healthy horses (Spencer et al, 2005). Given the inhibitory role of immune mediator TGFβ, on the effects of cytokine IFNγ, which plays a crucial role in protecting against *S. neurona* infection in mice, and resolving other intracellular infections, it was thought that EPM affected horses potentially expressed more TGFβ than normal healthy horses. It was found that EPM affected horses (n=9) had decreased concentration of CSF TGFβ compared to healthy horses (n=9) but there was great variance in sample concentrations potentially a consequence of duration EPM affected horses had been clinically affected (Furr and Pontzer, 2001). *In-vitro* experiments demonstrated that CSF collected from horses does have immune regulatory properties as treatment with anti- TGFβ antibody did result in an overall increase in IFNγ production in both control and EPM horses (Furr and Pontzer, 2001). When comparing neurologic and non-neurologic horses with *S.
neurona CSF antibodies, Njoku et al, (2001) discovered that non-neurologic horses had greater CSF concentration of nitric oxide metabolites compared to neurologic horses. These results suggest that nitric oxide metabolites may play role in resolving S. neurona infection in exposed horses. Conversely, in mouse models which are either deficient for inducible nitric oxide synthetase (iNOS) or endothelial nitric oxide synthetase (eNOS) S. neurona encephalitis is not inducible (Rosypal et al, 2002) but the disease susceptibility in mice lacking the neuronal isoform of nitric oxide synthase (nNOS) was not investigated. Therefore, it is possible that nNOS plays a role in S. neurona infection resolution as suggested by Njoku et al (2001).

While more is known about the immunopathogenesis of EPM than when investigations began decades ago, much remains unknown. It is still not well established how S. neurona is able to migrate into the CNS after clinical EPM horses are exposed to S. neurona. Likewise, it is unknown if exposed horses which remain subclinical mount the appropriate immune response necessary for clearance of the infection without insignificant damage to the CNS or, if S. neurona never enters the CNS in this population of horses. The original experimental horse model studies utilizing dexamethasone and transport to induce immune suppression reported that neurologic abnormalities are mild and in some cases are beginning to resolve by the end of the study period. Likewise, establishment of replication outside of the CNS seems to be necessary for the development of clinical neurologic disease as direct inoculation of S. neurona merozoites into the CNS did not result in neurologic disease. However, the horses in this study which had neither sera nor CSF antibodies against S. neurona prior to infection did develop antibodies against the parasite demonstrating an immune response (Lindsay, 2000).
While the experimental model developed by Ellison et al (2004) eliminated the immune suppression component of other models, which hampered the ability to accurately investigate the immune response, the model bypasses the natural infection route of gut which also proposes an issue.

**Mouse model**

In the time paralleling the investigations of researchers working to establish an experimental horse model to study EPM, researchers sought a smaller model to recapitulate EPM like disease. Marsh et al (1997) was of the first investigators to report that immune compromised mice lacking T lymphocytes (C57Bl/6 nude), succumbed to EPM like disease after infection with *S. neurona* merozoites. Following this investigation Dubey and Lindsay (1998) discovered robust *S. neurona* protozoal encephalitis in Balb/c interferon gamma (*Ifnγ*-/−) mice, initiating the common use of this mouse as a model for *S. neurona* infection and neurologic disease. Both the Balb/c and C57Bl/6 mouse strains with the *Ifnγ* gene knockout develop neurologic abnormalities on average within 30 days post infection, and commonly serve as positive control mice in investigations employing other immune compromised mice (Rosypal et al, 2002, Witonsky et al, 2003, Witonsky et al, 2005a, Witonsky et al, 2005b, Hay et al, 2019). Infection of Balb/c *Ifnγ* −/− mice with *S. neurona* sporocysts provided awareness on the migration and replication pattern, in a neurologic disease susceptible host. Initially, replication occurs in the small intestine, followed by the mesenteric lymph node, limited replication occurs in the visceral tissues and by the 3rd week post infection replication occurs almost strictly in the CNS, although *S. neurona* is found in the brain 11 days after infection (Dubey, 2001d). The *Ifnγ* −/− mouse has served as a proof of concept model for effective treatment options for *S.
neurona infection (Lindsay and Dubey, 2001, Dubey, 2001, Franklin et al, 2003) and the potential of treatment failure related to disease relapse in EPM affected horses (Hay et al, 2019). Additionally, neurologic disease will result in Ifny -/- mice that are infected with both S. neurona sporocysts by oral or subcutaneous inoculation and merozoites by subcutaneous inoculation (Dubey, 2001). At least 1000 merozoites are needed for consistent infection and antibody production, and at least 1000 sporocysts provides the most consistent and robust infection (Dubey, 2001). While many S. neurona isolates are infective to Ifny gene knockout mice, viability of sporocysts and merozoites in inoculums (Cheadle et al, 2001c) and for some isolates, and extensive passage negatively alters virulence and pathogenicity (Dubey, 2001).

Mouse model Immunopathogenesis
The robust susceptibility of the Ifny -/- mouse to S. neurona highlights the essential role of this cytokine in disease protection but exactly how it exerts protection is not easily answered, as this pleiotropic cytokine has an array of functions and is produced by a variety of both innate and adaptive immune cells. The B6.129P2- β2m^tm1/unc^ knockout mouse, void of MHC I expression and consequently lacking IFNγ producing CD8 + T lymphocytes, developed encephalitis as a result of S. neurona infection (Witonsky et al, 2005a). These mice didn’t develop encephalitis until 150 DPI which was at a significantly later time point that B6 Ifny -/- positive control mice (Witonsky et al, 2005a). These results may indicate that the cytokine IFNγ may be more essential for protection against S. neurona induced encephalitis than CD8+ T lymphocytes.

Interferon gamma induces nitric oxide synthetase (NOS) as defense mechanism against invading intracellular pathogens but genetic depletion of inducible NOS or
endothelial NOS in mice, did not yield encephalitis in *S. neurona* infected mice (Rosypal et al, 2002). Interestingly, *S. neurona* encephalitis is only observed in lymphocyte null ICR *scid* mice (Marsh et al, 1997) when IFNγ is depleted (Sellon et al, 2004), despite them lacking the seemingly protective role of T lymphocytes. This was refuted by Ahlgrim et al (unpublished work) who reported that Balb/c mice with the *scid* mutation displayed resistance to *S. neurona* infection but C57Bl/6 *scid* mice were not. These discrepancies suggest a potential genetic predisposition to *S. neurona* protozoal encephalitis, potentially relating to MHC haplotype, which differs amongst the ICR, Balb/c and C57Bl/6 mouse and further investigation on this topic matter is warranted. Additionally, as it is known that the majority of *S. neurona* exposed horses do not develop clinical neurologic disease but elicit an immune response resulting in antibodies against *S. neurona*, the role of B lymphocytes was investigated in the mouse model. It was found that mice lacking B lymphocytes (B6.129S2-Igh-6tmlcgn/J) remain neurologically normal for as long as 240 DPI with *S. neurona* (Witonsky et al, 2005b). The immune compromised mouse has served as a reliable and informative model for investigating EPM but translation of results to the horse should be done cautiously.

**Conclusions**

The results from the investigations pertaining to *S. neurona* biology, EPM immunopathogenesis, mouse and horse experimental models, which have been conducted over the past several decades, have improved the understanding about EPM and treatment of the disease. However, much of the clinical issues that still exist relating to EPM concern poor response to treatment resulting in disease progression to the point of
euthanasia being the most humane option for the horse. These issues could be addressed by further understanding of the immune response or further exploration of treatments mentioned in this literature such as bump kinase inhibitors, that are not regularly used for treatment now, or new therapies such as immune modulators. An immunomodulator such as Zylexis (inactivated parapoxovis virus) has demonstrated to increase INFγ (Horohov et al, 2008) which is involved in the protective immune response against S. neurona. Future studies that investigate the immunopathology or immunopathogenesis of EPM would ideally have large sample populations in order to investigate the diversity associated with different disease states, acute, chronic and reoccurring and treatment response. A study involving a large sample population would need to be a collaborative effort of researchers to gather enough samples from naturally affected EPM horses and healthy control horses, which would minimize issues associated with expenses and ethics of experimental EPM study models. Future mouse studies could include infection models which evaluate disease susceptibility based on MHC haplotype to further investigate the role of a genetic predisposition. Likewise, a study evaluating the MHC haplotype in EPM affected horses could also provide valuable information as it is probable that there is a genetic predisposition in horses. A genetic mutation of the interferon gamma gene, interferon gamma receptor gene or T-bet gene could also explain EPM susceptibility. A T-bet mutation could decrease IFNγ production by CD8 + T lymphocytes, impair T lymphocyte antigen priming, and decrease the cytotoxic function of natural killer cells. These immunological impairments have been observed in EPM affected horses. Additionally, further studies investigating the role of resident CNS immune cells such as astrocytes and microglia in S. neurona infection would also be beneficial. These resident CNS immune
cells play important roles in both infectious and degenerative disease pathogenesis in humans and therefore may also be important in horses (Lyman et al, 2014).

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Chapter III: *Saracocystis neurona* induced myeloencephalitis relapse following anticoccidial treatment

HAY ET AL. – *S. NEURONA* INFECTION RELAPSE POST TREATMENT

*SARACOCYSTIS NEURONA*- INDUCED MYELOENCEPHALITIS RELAPSE FOLLOWING ANTICOCCIDICAL TREATMENT

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ABSTRACT

*Saracocystis neurona* is a ubiquitous parasite in the eastern United States, which is the principal causative agent in the neurologic disorder, equine protozoal myeloencephalitis (EPM). While much is known about this protozoa’s life cycle in its natural host, the opossum (*Didelphis virginiana*), little is known of how it acts in the aberrant equine host which displays a high incidence of exposure with a relatively low rate of morbidity. For this study, we employed the popular interferon gamma knock out mouse model to determine the potential for recrudescence of *S. neurona* infection after treatment with the anticoccidial drug, diclazuril. Mice were infected with *S. neurona* merozoites and 7 days post infection (DPI) they were treated with diclazuril for 30 or 60
days or not treated at all. All infected non-treated mice developed neurologic signs consistent with *S. neurona* infection within 30 DPI. All diclazuril treated infected mice remained clinically normal while on treatment but developed neurologic signs within 60 days of treatment cessation. Histological examination of cerebellums from all infected mice demonstrated characteristic lesions of *S. neurona* infection, regardless of treatment status. Cerebellar samples collected from infected treated mice, displaying neurologic signs, produced viable *S. neurona* in culture. However, cerebellar samples collected from infected and neurologically normal mice at the end of a 30 day treatment period, did not produce viable *S. neurona* in culture. Analysis of the humoral immune response in infected mice showed that during treatment IgM antibody production decreased suggesting the organism was hidden from immune surveillance. The cessation of treatment and subsequent development of neurologic disease resulted in the increase of IgM antibody production suggesting re-recognition by the immune system. Based on the study results the authors propose that diclazuril was able to inhibit the replication and migration of *S. neurona* but not fully eliminate the parasite suggesting recrudescence of infection after treatment is possible.

**KEY WORDS**

Diclazuril, *Saracocystis neurona*, Equine Protozoal Myeloencephalitis, Relapse, Horse, Opossum, *Didelphis virginiana*

Equine protozoal myeloencephalitis (EPM) is the most prevalent protozoal disease of horses in North America with the predominant cause being ingestion of *Saracocystis neurona* sporocysts deposited by opossums (*Didelphis virginiana*) (Dubey
The resulting neurological disorder affects approximately 14 of every 10,000 horses older than 6 mo of age in the United States (NAHMS, 2001). The reason for high exposure rates but relatively low morbidity remains unclear as does the life cycle of the parasite in the aberrant horse host. Previous studies indicate a potential aberrant immune response to *S. neurona* in susceptible horses (Spencer et al., 2004, Scott et al., 2005, Yang et al., 2006, Lewis, 2014), indicating EPM susceptibility may persist for the life of the animal and is not a onetime occurrence. Clinical treatment consists of the use of anti/protozoal drugs such as ponazuril, diclazuril and sulfa drugs as well as corticosteroids to combat associated inflammation (Reed et al, 2016). Based on Saville et al. (2000) study, which included all stages of disease as well as all different treatments, the average equine patient is expected to improve only one neurological grade. Approximately 10-25% of EPM affected horses that respond positively to appropriate treatment are at risk for developing recurrent or novel neurological signs after discontinuing therapy. Whether the horses responding to treatment, are relapsing from their initial infection (i.e. persistent infection) or from re-exposure to the environmental stages of *S. neurona* is not fully understood (MacKay et al., 2006). Challenges of using the horse to study EPM are numerous and include the complexity of experimental infection, the terminal end point nature of many studies, and the difficulty in controlling environmental factors. (Saville, 2001, Witonsky, 2008, Lewis, 2014) Alternatively, immune compromised, C57bl/6J or BALB/c interferon gamma gene knockout (*Ifnγ* −/−) mice offer a regularly utilized model for addressing certain aspects of *S. neurona* pathology (Dubey and Lindsay, 1998; Dubey., 2001, 2013; Witonsky et al., 2003). The goal of the current study was to determine the potential for
persistent infection by *S. neurona* in mice after receiving diclazuril treatment previously shown to inhibit disease in this model (Dubey et al., 2001).

**MATERIALS and METHODS**

*Saracocystis neurona* culture and preparation of inoculum

Merozoites of the sixth passage of the Sn-15OP isolate were grown in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 (ATCC CCL-70)) American Type Culture Collection, Manassas, Virginia, separated from host cells, collected and enumerated for inoculations as previously described (Lindsay et al., 2013). The Sn-15OP isolate was isolated from the brain of an *Ifnγ−/−* mouse fed sporocysts obtained from the feces of a naturally infected opossum from Virginia (Dubey, 2000). This isolate was originally a gift from Dr. J. P. Dubey, United States Department of Agriculture, Beltsville, Maryland and has been maintained cryopreserved in liquid nitrogen.

**Mice**

Male and female B6.129S7-*Ifng*<sup>−/−</sup>/J (B6.*Ifnγ−/−*) mice, age 8-10 wk old, were obtained from Jackson Laboratory (JR2287, Bar Harbor, Maine). Mice were maintained at Virginia Tech in accordance with an Institutional Animal Care and Use Committee (IACUC) breeding protocol and experimental studies were conducted under other IACUC approved protocols. There were three experimental mouse groups that were infected with *S. neurona* merozoites: infected untreated (n=13), 30 day treatment (n=15) and 60 day treatment (n=12). Mice in the treatment groups were fed rodent diet compounded with diclazuril for the duration of 30 or 60 days beginning 7 days post infection (DPI). At the end of the 30 or 60 day treatment period, mice were changed to
the control diet for the 60 day observation period. Another group of mice did not receive treatment and remained uninfected (naïve, n=9). Another group of mice was fed diclazuril chow for 60 days and not infected (medicated diet, n = 5). The final group of infected, untreated mice received control diet.

For infection, mice were injected subcutaneously with $2 \times 10^6$ *S. neurona* merozoites. Blood was collected from the submandibular vein from live mice for sera antibody analysis at 7 days post infection (DPI) and on the final day of 30 day treatment. Post euthanasia blood was collected via cardiac puncture for sera antibody analysis. Mice were euthanized using CO$_2$ asphyxiation when clinical neurological signs relating to lack of balance and coordination were observed.

**Table I: Mouse group, treatment, sample collection and neurologic sign development.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Infected (Day 0)$^a$</th>
<th>Treatment diet (7DPI)$^c$</th>
<th>Sample collection: serum and cerebellum</th>
<th>Neurologic sign development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control (n=12)</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Positive Control (n=13)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes within 30 DPI</td>
</tr>
<tr>
<td>30 Day Treatment (n=15)$^b$</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes within 60 DPT</td>
</tr>
<tr>
<td>60 Day Treatment (n=12)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes within 60 DPT</td>
</tr>
</tbody>
</table>

$^a$ Mice infected with $2 \times 10^6$ merozoites subcutaneous injection.

$^b$ Serum samples collected Day 0, 7DPI and on the final day of 30 day treatment.

$^c$ Diet changed to control after treatment duration (30 or 60 days) control mice received control diet.

DPT= days post treatment
**Diclazuril treatment**

Diclazuril (Sigma-Aldrich, St. Louis, Missouri and LKT Laboratories, St. Paul, Minnesota) was compounded into 5LG4 rat and mouse chow (Test Diet, St. Louis, Missouri) at a concentration of 50 ppm. Medicated chow provided 10mg of diclazuril/kg of body weight to each mouse, a dose previously shown to prevent the development of *S. neurona* associated disease in *Ifnγ*-/ mice (Dubey et al., 2001). The rat and mouse chow (5LG4), without diclazuril served as the control diet.

**Cerebellum preparation and histology**

At the time of euthanasia, cerebellums were collected and those intended for the use of histology, were fixed in 10% buffered formalin solution. The cerebellum was then embedded in paraffin and processed for hematoxylin and eosin (H&E) staining. Triplicate sections were cut from paraffin blocks and used for immunohistochemical staining. One sample section was assessed for the total number of perivascular cuffs, gliosis foci and number of organisms for each cerebellum collected and given a score of 1 (mild) - 6 (severe). The total number of perivascular cuffs and gliosis foci increased with score where a score of 1 = 0-10 cuffs or foci and 6 = 55-70 cuffs or foci. The total number of organisms was scored in a similar manner, score 1 = 0-20 organisms and 6 = number organisms >100. Sections of cerebellum were stained with polyclonal rabbit anti-*S. neurona* anti-sera as described by Gerhold et al. (2005) with the exception that sections were digested with perxo-block (Novex, Life Technologies, Carlsbad, California) prior to incubation with anti-sera at 1:500 dilution.

**Sera immunoglobulins**
Blood was collected from infected untreated (n = 10), naïve (n = 9) and 30 day (n = 10) and 60 day (n = 10) treatment groups at the time of euthanasia, for sera IgM, total IgG and S. neurona immunoglobulin analysis by enzyme linked immunoabsorbent assay (ELISA). Serum was collected from whole blood, following centrifugation and stored at -20°C until utilized for ELISA. Immunoglobulin ELISAs were performed as described (Christianson et al., 1997) using IgG and IgM isotypes (Southern Biotech, Birmingham, Alabama). Plates were read on an Infinite M200 Pro plate reader with Magellan 7.0 software (Tecan, Mannedorf, Switzerland).

For S. neurona specific ELISA, a S. neurona merozoite protein lysate was created as a plate coating agent for antibody capture. For the lysate, frozen Sn-15OP merozoites were thawed and centrifuged at 2,500 g for 5 min at 4°C, merozoites were washed twice in ice cold 1x PBS, re-suspended to a concentration of 1x 10⁷/mL in 1x PBS, 10 mLs of radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, Massachusetts) was added per 1mL of merozoite suspension. The merozoite suspension was incubated on ice for 15 min with intermittent manual shaking and then centrifuged at 1,400 g for 15 min at 4°C. After centrifugation, the supernatant was collected and 100 uL was used to coat the ELISA plate wells (Thermo Fisher, Ref # 9018) incubating overnight at 4°C. The protocol then proceeded as described in Christianson et al. (1997). The detection antibody was goat anti mouse-Ig kappa- alkaline phosphatase (Southern Biotech) and optical density (OD) values were reported for data analysis.

**Cerebellum culture and observations**

At the time of euthanasia cerebellums intended for the use of culture, were collected from infected 30 day treated mice at the following time points: on the final day
of treatment (n=3), 12 days post cessation of treatment (n=3) and at the time of neurologic sign development post treatment (n=2). Additionally, cerebellums were collected from infected untreated mice (n=2). Immediately after collection cerebellums were placed in ice cold 1xPBS and then homogenized in 1 ml Hanks balanced salt solution (HBSS) using a sterile Teflon-coated tissue grinder and 12 ml grinding tube. The homogenized brain-HBSS solution was inoculated on to 2 (25 cm²) tissue culture flasks containing monolayers of CV-1 cells. The homogenate was incubated with CV-1 cells for 2 hr at 37° C. After 2 hr, the inoculum was removed, the cell monolayers were washed with HBSS, and 5 ml of maintenance medium was added for long term culture. Cultures were examined twice weekly and the medium replaced twice weekly for a month and then once weekly thereafter until *S. neurona* was detected or for 2 mo when the study was ended, and cultures were considered negative.

**Statistical analysis**

Neurologic disease incidence was determined using a log rank Mantel-Cox test with a significant level set at P < 0.05 and Bonferroni correction to account for multiple comparisons with a significance threshold at P < 0.017. Significance for immunoglobulin changes during treatment was determined using ANOVA accounting for repeated measures and multiple comparisons with Tukey’s T-test. To assess the immunoglobulin changes that occurred between the naïve mouse group and throughout treatment ANOVA was used with Dunnetts test to account for multiple comparisons. All other statistical analysis was calculated using Kruskal- Wallis test and Dunn’s test for post hoc comparisons. The significant level was set at P < 0.05. All analyses were performed using GraphPad Prism software version 6 for Mac (GraphPad Software, La Jolla, California).
RESULTS

*Saracocystis neurona* induced myeloencephalitis

All infected untreated mice developed neurologic signs 23 days of starting the non-medicated diet. *Saracocystis neurona*-infected diclazuril-treated mice did not develop clinical signs while on the medicated diet. Both the infected 30 and 60 day treated mice remained clinically normal on the non-medicated diet for significantly longer than the infected untreated mice. Neurologic disease did occur within 60 days of ceasing diclazuril treatment in all infected treated mice (Table I). There was no statistically significant difference in the incidence of neurologic disease between the 30 day treatment and 60 day treatment groups after switching to the control diet (Fig. 1).

![Figure 1](image)

Figure 1. The graph depicts the percent of neurologically normal mice versus the number of days mice have been consuming control diet. Time point day 0 represents the day mice were placed on the control diet. The infected untreated mice started consumption of non-medicated diet 7 DPI, and 30 and 60 day treated mice started at the end of the treatment periods. Closed circles represent number of neurologically normal mice at that time point within each group. **** P = <0.0001.
Histopathological analysis

Histology results confirm *S. neurona* induced encephalitis. When assessed at the time of neurologic sign onset, the cerebellums of infected untreated mice possessed significantly more gliosis foci by number (Fig. 2A) and perivascular cuffs (Fig. 2B) compared to infected 30 and 60 day treated mice. *Saracocystis neurona* organisms were found in significantly greater numbers in cerebellum sections from infected untreated mice compared to mice in the 60 day treatment group at the time of necropsy. There were no

**Figure 2.** Scores of histopathologic changes associated with infection and treatment (trt) in cerebellums collected from mice displaying neurologic signs. (A) perivascular cuffing foci in cerebellum H&E stained sections. (B) Gliosis foci in cerebellum H&E stained sections. (C) *Saracocystis neurona* organisms in H&E and IHC stained cerebellum sections. Male *B6. Ifng−/−* mice, age 8-10 wk at start of experiment. * P = <0.05, ** P = <0.005
significant differences in number of gliosis foci (Fig. 2A), perivascular cuffing (Fig. 2B)

Figure 3. Cerebellar immunohistochemistry and H&E images. All images are of cerebellum samples collected when neurologic signs were displayed by infected untreated mice (A, B) 30 day treated mice (C, D) and 60 day treated mice (E, F). (A) H&E image where the asterisk (*) indicates eosinophilic inflammation and arrows indicate Saracocystis neurona asexual stages. (B) H&E image where asterisks (*) indicate eosinophilic inflammation and arrows gliosis + eosinophilic inflammation. (C) IHC image arrows and dark brown staining indicate S. neurona asexual life stages. (D) H&E stained asterisks (*) indicate perivascular cuffing and arrows indicate gliosis. (E) IHC image arrows and dark brown staining are S. neurona asexual life stages. (F) H&E staining asterisks (*) indicate gliosis and eosinophilic inflammation and arrows S. neurona asexual life stages.
or *S. neurona* organisms (Fig. 2C) between the 30 and 60 day treatment groups. These results indicate that diclazuril treatment resulted in less severe cerebellar lesion. Although treatment did not impact sera IgM and IgG levels at the time of neurologic disease development it did result in a strong decrease in sera IgM levels before neurologic disease incidence. IgM levels trended upwards at 7DPI compared with naïve mice and then fell by the end of treatment (Trt End) with a significant elevation appearing at the time of neurologic sign development (Fig. 5A). Total IgG levels increased at the end of treatment and terminal time points with no significant difference between the Trt End and terminal time point (Fig. 5B). *Saracocystis neurona* specific antibody OD values become significantly elevated at the terminal time point (Fig. 5C).

**Cerebellum culture and observations**

**Table II: Cerebellum culture time point and organism retrieval**
Of 3 cerebellum samples collected on the final day of a 30 day treatment period and of three collected 12 days post treatment, no viable *S. neurona* was recovered after culture. However, of 2 cerebellum samples harvested from mice demonstrating neurologic signs within 60 days after a 30 day treatment period, one produced viable *S. neurona* that replicated in culture (Table II). Our results in these immunodeficient mice show that viable parasites can remain in the mice even after appropriate therapy with diclazuril.

**DISCUSSION**

Using the immunocompromised *Ifnγ*−/− mouse, the current study demonstrates that continuous diclazuril treatment for 30 or 60 days prevents the development of *S. neurona*.

<table>
<thead>
<tr>
<th>Cerebellum culture time point</th>
<th>End of 30 day trt</th>
<th>12 days post 30 day trt</th>
<th>Infected 30 day treated</th>
<th>Infected untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live Organism Retrieval</td>
<td>0/3</td>
<td>0/3</td>
<td>1/2</td>
<td>2/2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cerebellums collected from mice displaying neurologic signs
induced protozoal myeloencephalitis and associated neurologic signs. However, within 60 days after cessation of diclazuril treatment mice develop neurologic signs. This suggests that treatment prevents replication and/or migration of *S. neurona* but cessation of treatment leads to resumption of pathogenic activity. Previous in-vitro cell culture studies demonstrated that diclazuril limited the development of *S. neurona* merozoites, but parasite replication was observed after the removal of diclazuril from cell culture media. This also suggests that diclazuril only has an inhibitory effect on parasite activity (Lindsay and Dubey, 2000). In the current study, cerebellum samples collected on the final day of a 30 day diclazuril treatment period yielded no viable *S. neurona*. However, a cerebellum sample collected from a neurologic mouse post treatment yielded viable *S. neurona* capable of replicating in culture. The results of our histology data reveal positive *S. neurona* antibody staining in the cerebellum regardless of treatment duration. The decrease in the number of gliosis foci and perivascular cuffs in the treated mice compared to untreated mice suggests that treatment decreases the severity of *S. neurona* associated lesions. It should be acknowledged that Dubey et al. (2001), administered diclazuril compounded rodent diet for 39 days, to *Ifnγ* /-/- mice 7 days after infection with 1000 *S. neurona* sporocysts from the isolate Sn-15OP and sacrificed mice 8 days after treatment. They found that 8 days after the cessation of treatment, cerebellums subpassaged to naïve *Ifnγ* /-/- mice were not pathogenic nor did cerebellum histology results show positive *S. neurona* antibody staining (Dubey et al., 2001). In the current study, cerebellum samples collected 12 days after 30 days of treatment from infected mice did not yield *S. neurona* in culture suggesting that the brains were not yet parasitized at this point after treatment. We hypothesize that if treated infected mice in Dubey’s study were left alive for 60 days
post treatment, clinical disease may have developed, as in our study. Collectively, these results indicate *S. neurona* can resume its activity after cessation of diclazuril *in vivo* barring an adequate immunological response from the host.

While the immunologic response to *S. neurona* in *Infγ*−/− mice is not directly translatable to equine patients, quantifying the humoral response gives clues to immune awareness of parasitic infection. Extraneural *S. neurona* replication occurs 7 DPI (Dubey, 2001) which allows for antigen recognition by naïve B-lymphocytes resulting in the modest elevation of sera IgM concentrations 7 DPI compared to the naïve mouse group. At the end of 30 days of diclazuril treatment, sera IgM concentrations declined from the beginning of treatment (7 DPI) similar to those observed in the naïve mouse group suggesting that few new naïve B-lymphocytes were encountering parasite during treatment. The resumption of active *S. neurona* infection and parasite replication and migration is probable after treatment was discontinued as indicated by the significant increase in sera IgM concentrations at the time of neurologic disease development. The significant increase in total IgG sera concentration throughout the course of treatment can be attributed to maturation of the initial immune response, with IgM B-lymphocytes switching isotypes to IgG through the process of class switch recombination. The significant increase in *S. neurona* antibody OD values at the time of neurologic sign also supports maturation of an immune response to *S. neurona* and relates the humoral immune response to *S. neurona* infection. Pusterla et al. (2015) demonstrated that foals residing in a high risk EPM area treated with a low dose of diclazuril (0.5 mg/kg) had significantly less sera conversion of antibodies against *S. neurona* compared to untreated foals following the decline of maternal antibodies. Furr et al. (2006) also demonstrated
the prophylactic nature of the coccidiostat Ponazuril in decreasing the incidence and severity of experimentally induced EPM, assessed by sera and CSF antibodies and clinical neurologic signs. Murphy et al (2006), experimentally infected horses with *S. neurona* and demonstrated significant increases in *S. neurona*-IgM concentrations in serum and CSF samples collected from experimentally infected horses. The horses in the mentioned study developed clinical neurologic disease as a result of the experimental *S. neurona* infection. While our study was performed in the immunocompromised mouse model and cannot be directly correlated to disease in the horse, the previously mentioned equine model studies by Furr et al. (2006), Murphy et al (2006), Pusterla et al. (2015), also support the efficacy of humoral immune response analysis. The sera immunoglobulin results from the current study, also highlight the potential for evaluating *S. neurona* specific IgM antibodies clinically in equine patients, before the start of anti-protozoal treatment, at the time of treatment discontinuation and periodically after to monitor for the development of recurrent infection.

Recurrent disease in horses may be a result of persistent infection due to treatment failure as opposed to re-exposure to parasite. Other EPM treatment efficacy studies demonstrate potential treatment failure that could lead to disease relapse. Furr et al (2001) demonstrated that within 118 days after appropriate ponazuril treatment only 62% of horses in the study improved neurologically 1 grade and 10% of treated horses had cerebral spinal fluid negative for *S. neurona* antibodies by 90 days post treatment. In the same study 8% of horses that displayed improvement after treatment demonstrated recurrent disease by 118 days post treatment. Horses in the study were not housed in a manner to determine if recurrent disease could be attributed to re-exposure to *S. neurona*
or persistent infection due to treatment failure but it was hypothesized to be a result of
treatment failure (Furr et al., 2001). Additionally, horses treated with
sulfadiazine/pyrimethamine have a disease relapse rate of 25% (Fenger, 1998). To the
best of the authors knowledge, no studies have been done in horses that evaluate the
disease relapse rate related to diclazuril treatment. In many cases, the discussed anti-
protozoal drug coupled with an appropriate immune response in an otherwise normal
horse is a successful treatment strategy for clinically affected patients but for those
patients that present with recurrent signs, other treatment strategies should be considered.
The results of this study imply that diclazuril can inhibit S. neurona activity but not
eliminate the parasite, providing evidence that recurrent disease could be a result of
persistent infection and treatment failure and not simply reinfection.

ACKNOWLEDGMENT

Authors would like to thank Dr. Heather Bradford for her contribution to the
statistical analysis of the manuscript.

LITERATURE CITED

mice are protected from hypergammaglobulinemia and have defective antibody
responses because of increased IgG catabolism. Journal of Immunology 159:
4781–4792.


Chapter IV: The efficacy of decoquinate in preventing the development of neurologic disease in Saracocystis neurona infected C57Bl/6 interferon gamma knockout mice

Abstract
Equine protozoal myeloencephalitis (EPM), predominantly caused by the protozoa Saracocystis neurona, is a common neurologic disease of horses from North America. Exposure to the parasite occurs frequently but clinical neurologic disease presents in a small fraction of exposed horses. Treatment for EPM affected horses consists of anti-protozoal drugs along with anti-inflammatorries to combat associated inflammation in the central nervous system (CNS). Recently, the anti-protozoal drug decoquinate has been evaluated for its efficacy against S. neurona infection. In the current study, we sought to determine if decoquinate could retard neurologic disease in S. neurona infected C57Bl/6 interferon gamma knockout (B6Ifnγ/-) mice, an EPM mouse model. Decoquinate was administered to mice 7 days post infection, in the form of either medicated chow compounded with 30 or 60 ppm of decoquinate or by orally dosing mice daily with 20mg/kg of decoquinate. All treated mice developed neurologic signs within 30 days post infection (DPI) at a rate that did not significantly differ from infected untreated controls and S. neurona was found in the cerebellums of all infected mice regardless of treatment status. Additionally, mass spectrometry results indicate the greatest sera decoquinate concentration was found in mice from the group that received medicated chow with 60ppm of decoquinate opposed to the group that was orally dosed daily. Results of the current study indicate that decoquinate was not able to prevent neurologic disease in S. neurona infected B6Ifnγ -/-mice.

Introduction
Equine protozoal myeloencephalitis (EPM) is one of the most common neurologic diseases of horses in North America, with the principal basis for disease being ingestion
of *Saracocystis neurona* sporocysts. The pathogenic sporocysts are found in opossums (*Didelphis virginiana*) feces which contaminate the horse’s feed and water sources (Dubey and Lindsay, 1999, Dubey et al. 1999, Dubey et al. 2000). The prevalence of exposure to *S. neurona* is high but there is not a direct correlation between exposure incidence and clinical neurological disease incidence. Many neurologically normal horses test positive for *S. neurona* sera antibodies indicating exposure to the parasite. The reason for high exposure rates but relatively low morbidity remains unclear but previous studies indicate a potential aberrant immune response to *S. neurona* in susceptible horses (Spencer et al., 2004, Scott et al., 2005, Yang et al., 2006, Lewis et al, 2014). Clinical EPM treatment consists of the use of anti/protozoal drugs such as ponazuril, diclazuril and Sulfadiazine/pyrimethamine, as well as anti-inflammatory medications to combat associated inflammation in the CNS (Pusterla and Tobin, 2017). A study examining all stages of disease as well as all different treatment options, revealed that the average equine patient is expected to improve only one neurological grade with treatment. In the same study, approximately 10-25% of EPM affected horses that respond positively to treatment are at risk for developing recurrent or novel neurological signs after discontinuing therapy (Saville et al, 2000). This warrants investigation into novel treatment strategies to improve the prognosis for EPM affected horses, and recently, the anti/protozoal decoquinate has been examined for its efficacy as an EPM treatment.

A clinical field trial treating horses suspected to be affected by EPM with a compounded formulation of decoquinate and immunomodulator levamisole, found that clinical neurologic signs decreased after 10 days of treatment in treated horses (Ellison et al, 2012). Lindsay et al (2013) demonstrated that *in-vitro* decoquinate treatment inhibited
merozoite replication of two isolates of *S. neurona* merozoites, one opossum (SnOP-15) and one equine (Sn6) derived, and, at high enough concentrations, eliminated developing schizonts. While these two studies show promise for the use of decoquinate as an EPM treatment further studies with more defined and controlled study population, need to be conducted.

In the current study, we sought to determine the efficacy of decoquinate in preventing *S. neurona* induced encephalitis in the C57Bl/6 interferon gamma gene knockout (*Ifnγ*−/−), mouse model (Dubey and Lindsay, 1998; Dubey., 2001, 2013; Witonsky et al., 2003, Hay et al, 2019). Decoquinate is commercially available as a feed additive for livestock for treatment of coccidiosis in cattle, goats and poultry (Deccox, Zoetis). Currently it is compounded with immunomodulator levamisole and administrated as an oral paste once daily, for 10-day treatment period in EPM affected horses (Ellison et al, 2012 and Pathogenes, Reddick, Florida). In order to mimic commercially utilized decoquinate administration methods, two administration methods were tested in the current study; decoquinate medicated chow fed ad libitum and once daily oral administration by gavage. The mice were infected with *S. neurona* and treatment began 7 days post infection (DPI) with mice monitored for the development of neurologic signs to assess the ability of decoquinate to prevent persistent infection.

**Materials and Methods**

*Saracocystis neurona* culture and preparation of inoculum

Merozoites of the Sn-15OP isolate were grown in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1(ATTC CCL-70)) American Type Culture Collection, Manassas, Virginia), separated from host cells, collected and enumerated for inoculations
as previously described (Lindsay et al., 2013). The Sn-15OP isolate was isolated from the brain of an \textit{Ifn\textgamma} \textsuperscript{-/-} mouse fed sporocysts obtained from the feces of a naturally infected opossum from Virginia (Dubey, 2000). This isolate was originally a gift from Dr. J. P. Dubey, United States Department of Agriculture, Beltsville, Maryland and has been maintained cryopreserved in liquid nitrogen.

\textbf{Mice}

Male and female B6.129S7\textit{-Ifn\textgamma}^{\textsuperscript{m1Tb}J} (B6.\textit{Ifn\textgamma} \textsuperscript{-/-}) mice, age 8-10 weeks old, were originally obtained from Jackson Laboratory (JR2287, Bar Harbor, Maine). A colony of B6. \textit{Ifn\textgamma} \textsuperscript{-/-} mice was maintained through inbreeding with a brother sister mating strategy and mice used in the current study were from this colony. Mice were maintained at Virginia Tech in accordance with an Institutional Animal Care and Use Committee (IACUC) breeding protocol and experimental studies were conducted under other IACUC approved protocols. For \textit{Sarcocystis neurona} infection, mice were injected subcutaneously with 2 \times 10^6 \textit{S. neurona} merozoites. Mice were euthanized using CO\textsubscript{2} asphyxiation when clinical neurological signs relating to lack of balance and coordination were observed. Blood was collected post euthanasia via cardiac puncture from infected mice and sera was collected after centrifugation and stored at -20 C for further analysis. Cerebellum samples were also collected for histological analysis from all infected mice, post euthanasia. The body weight of all mice was monitored throughout the study and percent body weight loss was calculated after euthanasia.

\textbf{Decoquinate Treatment}

\textit{Medicated Diet}
Decoquinate (Sigma-Aldrich, St. Louis, Missouri) was compounded into 5LG4 rat and mouse chow (Test Diet, St. Louis, Missouri) at a concentration of 30 and 60 ppm. The 5LG4) without decoquinate served as the control diet. The S. neurona infected mice were divided in to three different groups: 30ppm medicated diet (n=6), 60ppm medicated diet (n=8) or control diet (n=4 per treatment). The consumption of the appropriate treatment or control diet began 7 days post infection (DPI) for 30 days. At the end of the 30-day treatment feeding period, mice were switched to the control diet. The time point of 7 DPI was selected for commencement of treatment, as primarily extraneural replication of S. neurona is occurring at this time point (Dubey, 2001).

**Oral gavage decoquinate treatment**

Decoquinate was administered in the form of an 8% decoquinate formulation contributed by Dr. Siobhan Ellison (Pathogenes Inc, Reddick, Florida). This formulation of decoquinate is most soluble at a pH 9 and optimal solubility was necessary to achieve for oral gavage administration. Decoquinate was suspended in sucralose sweetened sterile water at pH 9. Treatment began 7 DPI and was administered every 24 hours at a dose of 20 mg/kg (n=10) and sucralose sweetened sterile water was given to the control mice (n=9) for 30 days or until the development of neurologic disease.

**Cerebellum preparation and histology**

At the time of euthanasia, cerebellums were collected from mice treated by oral gavage (n=5), 60ppm decoquinate chow (n=5) and control mice (n=5) from both treatment trials (Table I). Cerebellum samples from mice treated with 30ppm of medicated chow were not processed since neither treatment option yielded protection against development of neurologic disease. It was opted to only select 60ppm medicated chow samples for
Cerebellums were fixed in 10% buffered formalin solution, paraffin embedded and sectioned for immunohistochemistry (IHC) staining. Sections of cerebellum were stained with polyclonal rabbit anti-\textit{S. neurona} anti-sera as described by Gerhold et al. (2005) with the exception that sections were digested with perxo-block (Novex, Life Technologies, Carlsbad, California) prior to incubation with anti-sera at 1:500 dilution. The sections were reviewed for positive \textit{S. neurona} staining with a Primo Star Upright Light Microscope (Carl Zeiss) to confirm \textit{S. neurona} presences in the cerebellum.

**Table I: Sample collection and processing for each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>Neurologic signs</th>
<th>Cerebellum(^a) Processing</th>
<th>IHC Positive(^b)</th>
<th>Mass Spectrometry(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected Untreated – 30ppm medicated diet (n=4)</td>
<td>Yes (4/4)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Infected Untreated – 60ppm medicated diet (n=4)</td>
<td>Yes (4/4)</td>
<td>Yes (n=2)</td>
<td>2/2</td>
<td>n= 2</td>
</tr>
<tr>
<td>Infected Untreated – oral gavage (n=9)</td>
<td>Yes (9/9)</td>
<td>Yes (n=3)</td>
<td>3/3</td>
<td>n=3</td>
</tr>
<tr>
<td>30ppm medicated Diet (n=6)</td>
<td>Yes (6/6)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>60 ppm medicated diet (n=8)</td>
<td>Yes (8/8)</td>
<td>Yes (n=5)</td>
<td>5/5</td>
<td>n=5</td>
</tr>
</tbody>
</table>
Oral gavage  (n=10)  Yes  (10/10)  Yes  (n=5)  5/5  n=5

a Number of collected cerebellum samples processed for each group.
b Number of processed cerebellum samples which were IHC positive for *S. neurona*.
c Number of sera samples processed for decoquinate Mass Spectrometry

**Mass spectrometry**  To ensure that decoquinate was delivered by both means of administration, mass spectrometry was performed using a LC/MS system and the protocol previously described (Wang, 2017) to detect and quantitate decoquinate in mouse sera. The sample size was 50 uL of sera and Decoquinate D5 (Sigma Aldrich, St. Louis, Missouri) was used for the internal standard. Sera samples collected from mice treated with 30ppm of medicated chow were not processed since neither treatment option yielded protection against development of neurologic disease. It was opted to select only 60ppm medicated chow samples for processing. The negative control samples were from mice which received control diet (n=2) or mice orally dosed with sucralose sweetened sterile water (n=3).

**Statistical analysis**

For analysis of the neurologic incidence curves, a log rank (Mantel-Cox) test was performed. For analysis of the mass spectrometry results, one-way ANOVA was conducted, and Tukey’s T-test accounted for post hoc multiple comparisons. A Pearson correlation test was conducted to assess correlation of weight loss and sera concentrations of decoquinate. All statistical analysis was done in Graph Pad Prism software and statistical significance was set at P<0.05.
Results

Neurologic incidence

Neurologic signs developed in all infected mice regardless of treatment status within 30 DPI and 23 days post treatment (DPT).

Neither decoquinate compounded chow (30 or 60ppm) or oral gavage administration of decoquinate were able to avert neurologic abnormalities and neurologic incidence was 100% in all infected B6Ifnγ-/- mice.

There were no significant differences in occurrence of neurologic sign development between the infected untreated control groups and 30ppm chow, 60ppm chow or

Figure 1: Neurologic incidence curves for all treatments compared to infected untreated controls for each treatment. All treatments began 7 DPI. A & B. Infected B6 Ifnγ -/- received 30 ppm compounded chow (A) or 60 ppm (B). C. Infected treated B6 Ifnγ -/- received oral administration (gavage) of decoquinate.
daily oral dose, conclusively implying no treatment effect (Figure 1A-C).

**Immunohistochemistry**

For each processed sample (Table I), two replicate cerebellar sections were examined for the presences of *S. neurona*. In all examined samples, positive IHC staining was found regardless of treatment status (Figure 2). The positive IHC results from the cerebellum samples collected from mice displaying neurologic abnormalities further support the inability of decoquinate treatment to prevent neuroinvasion and *S. neurona* replication in the CNS.

![Figure 2](image-url)

*Figure 2:* Representative immunohistochemistry images of cerebellum samples collected from A) infected untreated B6 *Ifnγ−/−* mouse B) oral gavage treatment and C) 60ppm medicated chow treatment. All cerebellums had positive *S. neurona* staining indicated by dark brown staining in images.
Mass spectrometry

The low solubility of decoquinate combined with the high sensitivity of the mass spectrometer used for sample processing led to detection of residual decoquinate in negative control samples which did not contain any decoquinate. Relative to the negative control samples collected from infected untreated mice, there were no significant differences in decoquinate sera concentrations in the 60ppm medicated diet or daily orally dosed treated mice. The sera samples collected from the oral gavage treated mice contained minimal amounts of decoquinate (Figure 3A). A portion (n=2) of the samples from mice which were treated with 60ppm medicated diet, contained decoquinate at sera concentrations notably greater than those obtained from the negative control mice (Figure 3A). Additionally, the sera concentration of decoquinate was not significantly correlated with any weight loss (percent body weight loss) which may have occurred during the infection time period, suggesting that decoquinate treatment had no effect on body weight loss (Figure 3B). It was not feasible to optimize the mass spectrometry protocol to account for residual decoquinate error due to limited sample concentration (ppb)

Figure 3: A) Serum concentrations of decoquinate measured by mass spectrometry. No significant differences were found relative to the negative control (control) samples. Data is presented as +/- SEM. B) A correlation plot of percent body weight loss correlation with sera concentrations of decoquinate. Increase body weight loss was not significantly correlated with increase in sera decoquinate concentration.
amount. Even though the majority of analyzed sera samples contained little to no decoquinate, there were several samples that contained concentrations above those from the negative control indicating that treated mice did receive decoquinate during the treatment periods.

**Discussion**

In the current study we found that when *S. neurona* infected B6 *Ifnγ*-*/-* mice were treated 7 DPI with mouse chow compounded with either 30ppm or 60ppm of the antiprotozoal drug, decoquinate, neurologic disease was not prevented in the mice. Likewise, oral administration of decoquinate at a dose of 20mg/kg once daily did not retard neurologic disease. All treated, infected mice developed neurologic signs similar to infected untreated mice within 30 DPI and *S. neurona* was identified by IHC in collected cerebellum samples, regardless of treatment status. Hay et al (2019) previously demonstrated that mouse chow compounded with 50ppm of the antiprotozoal diclazuril, an agent commonly used for EPM treatment, delayed the occurrence of neurologic disease in *S. neurona* infected B6 *Ifnγ*-*/-* compared to infected untreated controls. If the efficacy of decoquinate was to be consider comparable or superior to that of other EPM treatment options such as diclazuril, similar or better treatment efficacy would have been expected in the current study. It should be noted that the lipophilic properties of decoquinate do make it a difficult drug to pharmacologically compound, unlike diclazuril, proposing a concern that it was not effectively compound into the medicated diet used in the current study. This concern was addressed by performing mass spectrometry in efforts to assess administration success of decoquinate. Decoquinate was found in concentrations well above negative control mice concentrations in 2 of 5 mice.
fed decoquinate compound chow at 60ppm, which ensured ingestion of decoquinate from compounded chow. As to why decoquinate was not found in high concentrations of all analyzed mice in the 60ppm treatment group, this may be explained by the rapid clearance of decoquinate. Initial studies investigating the clearance of decoquinate from blood found the clearance to be rapid with nearly all drug cleared within one hour of dosing in investigated species which included, sheep, chickens, quail and cattle (Mitchell, 1988). It is unknown when the last time chow consumption (decoquinate intake) occurred in neurologic mice prior to euthanasia and blood collection. In the current study the two mice with the highest decoquinate sera concentrations experienced no weight loss, suggesting that decoquinate intake was not affected by neurologic deficits and perhaps intake of decoquinate occurred shortly before blood collection in these mice. Furthermore, the three mice from the 60ppm chow group, which experienced the greatest percent of body weight loss, had sera decoquinate concentrations very similar to those of negative controls. In these three mice, chow intake (decoquinate) may have been impeded by progression of S. neurona infection and this offers potential explanation for the low sera decoquinate concentrations.

To help combat the bioavailability issues of standard decoquinate, a specially formulated 8% decoquinate compound contributed by Dr. Ellison (Pathogenes Inc), was also tested in the current study. The formulation of decoquinate is similar to the one described by Wang et al (2013) which was formulated in a manner to reduce decoquinate particle size and increase solubility in order to increase bioavailability to improve its potential as an anti-malarial drug. In the current study, the 8% decoquinate formulation was orally administrated at a dose of 20mg/kg of decoquinate once daily by oral gavage.
technique. Our mass spectrometry results indicate that there were only slight, insignificant differences in the amount of decoquinate in the sera from the mice treated by oral gavage compared to the negative control samples. As previously mentioned, decoquinate is rapidly cleared after administration and this provides a plausible explanation as to why minimal amounts of decoquinate were detected in sera samples collected from the gavage mice. The last decoquinate dose the mice received was 24 hours prior to sera sample collection. The quick metabolism of decoquinate makes toxicity a low risk even when given frequently also resulting in little systemic accumulation supporting the need for frequent dosing. The overall results of this study question the efficacy of decoquinate as an EPM treatment.

Currently, there are no published decoquinate pharmacokinetic studies in horses and whether the drug is able to pass the blood brain barrier and enter into the CNS, remains unknown. In Ellison et al (2012), clinical field trial suggested efficacy of compounded decoquinate and levamisole as successful treatment for presumptive EPM affected horses, the two compounds were not evaluated individually and in-vivo effects of each compound in the horse is unknown. *In-vitro* synergistic effects of decoquinate and levamisole were not observed (Lindsay et al, 2013) but this may differ *in vivo*. The results of the current *S. neurona* infection mouse model study demonstrates that decoquinate does not have the same properties in vivo as other available treatments such as diclazuril, despite promising *in-vitro* studies (Lindsay et al, 2013). This is most likely due to unfavorable biological properties of decoquinate, which result in decreased bioavailability. Based on the results of the current study and the gap in knowledge about
decoquinate pharmokinetics in the horse, further studies need to be conducted to ensure validity of decoquinate as a treatment option for EPM.

Acknowledgments

Dr. Siobhan Ellison is thanked for her contribution of the decoquinate formulation used in the oral gavage experiments in this study.

References


Ellison, S.P. and Lindsay, D.S. 2012. Decoquinate combined with levamisole reduce the clinical signs and serum SAG 1,5,6 antibodies in horses with suspected Equine Protozoal Myeloencephalitis. International Journal Applied Research Veterinary Medicine. 10


Chapter V: What the immunocompromised models tell us about the immunopathogenesis of S. neurona induce protozoal encephalitis.

Abstract

The equine neurologic disease, equine protozoal myeloencephalitis (EPM), is difficult to study in the horse and often mouse models are employed to investigate the disease instead. Throughout the late 1990’s to the present, immune compromised mouse models have been utilized to investigate the immunopathogenesis of S. neurona infection. The results of the studies reviewed here indicated that an IFNγ driven immune response, especially as it pertains to MHC expression, and T-lymphocyte mediated immunity, seems be of utmost importance, to prevent onset of clinical neurologic disease in S. neurona infected mice. The genetically engineered immune compromised mouse models which have been used to attempt to decipher the immune response which protects against S. neurona induced encephalitis are reviewed here.

Introduction

Equine protozoal myeloencephalitis (EPM) is caused by the apicomplexan protozoa Sarcocystis neurona which is deposited into the horse’s environment by opossum feces. Intake of contaminated feed or water exposes horses to S. neurona but most exposed horses never develop clinical neurologic disease associated with S. neurona infection in the CNS. However, 1% of the equine population in the United States (NAHMS, 2001) does progress to neurologic disease after S. neurona exposure and the seemingly aberrant immune response in this population is not fully characterized, nor is the immune response that negates neurologic disease after exposure in healthy horses. Throughout the late 1990’s to the present immune compromised mouse models have been used to investigate
the immunopathogenesis of *S. neurona* infection. The immunocompetent C57Bl/6 mouse does not develop neurologic disease upon infection with *S. neurona* but they do develop an immune response against the parasite, generating sera antibodies and a robust cell mediated immune response that seems to be driven by CD8 + T lymphocytes (Witonsky et al, 2003a). The utilization of these mouse models has provided insight into a protective immune response against *S. neurona* infection being mediated by interferon gamma (IFNγ) and T lymphocytes. The use of the mouse model has allowed for controlled and mechanistic studies and offered an alternative study model to help avoid challenges associated with the horse model, such as previous exposure and disease status of the horse, expenses and ethical concerns. In this review the contribution of the mouse model to elucidating the immunopathogenesis of *S. neurona* infection are highlighted.
<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Immune Dysfunction</th>
<th>Neurologic Signsa</th>
<th>Study Durationc</th>
<th>S.neurona Isolate</th>
<th>Reference</th>
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<tr>
<td>Balb/c</td>
<td>Immune Competent</td>
<td>No</td>
<td>50</td>
<td>SnOP-15 Sporocysts</td>
<td>Rosypal, 2002</td>
</tr>
<tr>
<td>Balb/c Ifnγ -/-</td>
<td>No IFNγ</td>
<td>Yes</td>
<td>30</td>
<td>SnOP-15 Sporocysts</td>
<td>Dubey and Lindsay, 1998</td>
</tr>
<tr>
<td>Balb/c scid</td>
<td>Lymphocyte null</td>
<td>Nob</td>
<td>60</td>
<td>SnOP-15 Merozoites</td>
<td>Hay, unpublished Ahlgrim, unpublished</td>
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<tr>
<td>C57Bl/6 (B6)</td>
<td>Immune competent</td>
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<td>240</td>
<td>SnOP-15 Sporocysts Merozoites</td>
<td>Rosypal, 2002 Witonsky, 2003a</td>
</tr>
<tr>
<td>B6 nude</td>
<td>No T-lymphocytes</td>
<td>Yes</td>
<td>50</td>
<td>UCD3 Merozoites SnOP-15 sporocysts</td>
<td>Marsh, 1997 Dubey and Lindsay, 1998</td>
</tr>
<tr>
<td>B6 scid</td>
<td>Lymphocyte null</td>
<td>Yes</td>
<td>35</td>
<td>SnOP-15 merozoites Unknown</td>
<td>Hay, Unpublished Ahlgrim, Unpublished</td>
</tr>
<tr>
<td>B6 Rag 1 -/-</td>
<td>Lymphocyte null</td>
<td>Yes</td>
<td>35</td>
<td>SnOP-15 Sporocysts</td>
<td>Hay, Unpublished</td>
</tr>
<tr>
<td>B6 iNos -/-</td>
<td>No inducible NOS or</td>
<td>No</td>
<td>64</td>
<td>SnOP-15 sporocysts</td>
<td>Rosypal, 2002</td>
</tr>
<tr>
<td>B6eNos -/-</td>
<td>No endothelial NOS</td>
<td>No</td>
<td>64</td>
<td>SnOP-15 sporocysts</td>
<td>Rosypal, 2002</td>
</tr>
</tbody>
</table>
I. Establishing Immunodeficient Mouse Model

After identifying the parasite responsible for neurologic disease in horses as *S. neurona* (Dubey et al, 1991) investigators sought to find a rodent model which could be employed

<table>
<thead>
<tr>
<th>Model</th>
<th>MHC I expression and CD8+ T lymphocytes</th>
<th>Development of neurologic signs as a result of <em>S. neurona</em> infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 β2m-/</td>
<td>Yes</td>
<td>30 DPI nr</td>
</tr>
<tr>
<td></td>
<td>SnOP-15 merozoites</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Witonsky, 2005a</td>
<td></td>
</tr>
<tr>
<td>B6 IgH-/</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>ICR scid</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UCD 2, 3 Merozoites and Sporocysts</td>
<td>Marsh, 1997</td>
</tr>
<tr>
<td></td>
<td>Opossum Sporocysts</td>
<td></td>
</tr>
<tr>
<td>NOD</td>
<td>Defective: Antigen presenting (DC), NK cell function, macrophage cytokine production, wound healing and C5 complement</td>
<td>135 SnOP-15 merozoites Hay, Unpublished</td>
</tr>
<tr>
<td>NOD Aicda -/-</td>
<td>See NOD + No CSR and SMH</td>
<td>135 SnOP-15 merozoites Hay, Unpublished</td>
</tr>
<tr>
<td>NSG</td>
<td>See NOD + no lymphocytes + dysfunctional NK cells</td>
<td>40 SnOP-15 merozoites Hay, Unpublished</td>
</tr>
</tbody>
</table>

Refers to average days post infection that neurologic signs developed if disease susceptible or duration of study if not disease susceptible

d Neurologic signs occur within 30 DPI

CSR= class recombination, SMH= somatic hyper mutation
to investigate the immunopathogenesis of *S. neurona* infection and potential treatment options. Marsh et al (1997) was of the first investigators to infect immunodeficient mice with culture derived *S. neurona* merozoites from the UCD 2 and 3 *S. neurona* strains. Three immunodeficient mouse models were utilized in this investigation, C57Bl/6 Foxn1<sup>nu</sup> Nu/J (B6 nude) which lack T-lymphocytes, ICR- Prkdc<sup>scid</sup> (ICR scid) and immunocompetent C57Bl/6 (B6) mice which received methylprednisolone acetate for immunosuppression. The ICR scid mice are lymphocyte deficient due to the protein kinase DNA activated catalytic polypeptide (Prkdc) mutation, which disrupts the maturation processes in lymphocytes. The B6 nude mice developed neurologic disease on average 50 days post intraperitoneal infection with 2x10<sup>5</sup> *S. neurona* merozoites derived from the equine *S. neurona* isolate UCD3. Histological analysis of the brains collected from these mice revealed encephalitis and presences of *S. neurona* and *S. neurona* was successfully isolated from cultured brain homogenates of infected B6 nude mice. In addition to the brain, other visceral tissue samples were collected from the B6 nude mice and the only other site of inflammation was in the liver. The ICR scid and immunosuppressed B6 mice did not develop neurologic disease following infection (Marsh, 1997). In this study mice lacking solely T-lymphocytes but intriguingly not those lacking both T and B-lymphocytes developed *S. neurona* induced encephalitis. This perhaps suggests that B-lymphocytes play a role in the pathogenesis or as suggested by Marsh et al, discrepancy in disease susceptibility may be a result of genetic differences. Nonetheless, a rodent model for EPM had begun to be established. Following this investigation Dubey and Lindsay (1998) infected B6 nude and Balb/c interferon gamma gene knockout (Ifng<sup>-/-</sup>) mice with opossum derived *S. neurona* sporocysts and
merozoites. It was reported, both B6 nude and Balb/c Ifnγ/- mice developed neurologic abnormalities attributed to S. neurona induced encephalitis, confirming disease susceptibility in B6 nude mice and proposing both another mouse model and the role of IFNγ, in the protective immune response against S. neurona infection. The Balb/c Ifnγ/- mouse presented a more economical mouse model to study EPM which also developed more severe S. neurona induced encephalitis compared to B6 nude mice and thus became the commonly used rodent model for studying EPM-like disease. The discovery of disease susceptibility in the Balb/ c Ifnγ/- or C57BL/6 Ifnγ/- mice, paved the way for rodent model investigations of S. neurona biology, treatments and immunopathogenesis. The repeatable and quick onset of neurologic disease in the Ifnγ/- mouse allowed it to be used as a positive control mouse to ensure pathogenicity of S. neurona sporocysts or merozoites of different isolates in the mouse model. They were also utilized as positive controls in investigations which assessed disease susceptibility in other genetically engineered, immune compromised mice.

II. The role of lymphocytes and MHC haplotype in the S. neurona disease susceptibility

In addition to Marsh et al (1997) findings of ICR scid mice being resistant to S. neurona induced encephalitis, Sellon et al (2004) also reported disease resistance in the ICR scid mouse unless IFNγ was depleted (see section: V). An unpublished (Ahlgrim et al) investigation reported when B6. CB17- Prkdc scid/SzJ (B6 scid) and CBBySmn. CB17 -
Prkdc scid/J (Balb/c scid) mice are subjected to *S. neurona* infection, only B6 scid mice are susceptible to protozoal encephalitis and Balb/c scid are resistant, much like the ICR scid mice. Due to the discrepancies of previous reports involving SCID mice the author of the current review infected three different lymphocyte null mice, B6 scid, Balb/c scid and B6.129S7-Rag1<sup>tm1Mom</sup> knockout mice (Rag1 -/-). The Rag1 -/- mutation is a complete null mutation of the recombination activating gene 1, which inhibits lymphocyte receptor maturation and consequently lymphocyte maturation and differentiation. The *Scid* mutation is spontaneous and can result in a leaky phenotype with the potential of minimal lymphocytes in circulation but this does not occur with the Rag1-/- . For this study the different lymphocyte null mice

**Figure 1:** Neurologic incidence plots with percent neurologically normal mice versus days post *S. neurona* infection. A. Comparisons of Balb/c scid mice, B6 scid and B6 *Ifnγ*-/- . B. Comparison of B6 Rag1 -/- mice and B6 *Ifnγ*-/- mice post C. Comparison of NSG mice and B6*Ifnγ*-/- mice. **** P < 0.0001, *** P <0.001 ** P <0.005 * P <0.05. All comparisons analyzed with Log -rank (Mantel-cox) test.
were infected with $2 \times 10^6$ *S. neurona* merozoites from the opossum isolate, Sn-OP15, via subcutaneous injection. After 60 days of observation the B6scid mice presented with neurologic signs at 35 DPI on average, Rag 1 -/- exhibited similar susceptibility but only one of 10 Balb/c scid mice presented with neurologic signs at 35 DPI, and the remainder of Balb/c scid mice were absent of neurologic signs at the conclusion of 60 day study period (Figure 1A &B). *Saracocystis neurona* protozoal encephalitis was confirmed by histology and *S. neurona* was found in the cerebellum of all mice that displayed neurologic signs (Figure 2 A&B).

One possible explanation for the susceptibility discrepancy in these three strains of SCID mice and *Rag 1* -/-, all lacking lymphocytes, is that the MHC haplotype differs between the four strains of lymphocyte null mice that have been infected with *S. neurona* (Table II). The MHC complex has both antigen recognition and presentation roles during an immune response and antigen recognition can vary based on MHC haplotypes. It is possible that a specific MHC haplotype (s) has greater recognition for *S. neurona* consequently, altering the immune response against *S. neurona*. Furthermore, a study which investigated protozoal encephalitis in both Balb/c *Ifnγ* -/- and B6 *Ifnγ* -/- reported the timing for the development of clinical neurologic disease to be prolonged in the Balb/c *Ifnγ* -/- compared to the B6 *Ifnγ* -/- especially as the amount of sporocysts used for inoculation decreased (Dubey et al, 2013). These results further support the possibility that MHC haplotype plays a role in *S. neurona* encephalitis susceptibility. However, if resistance to *S. neurona* encephalitis was solely dependent MHC haplotype then susceptibility differences would be expected in fully immunocompetent C57BL/6 and Balb/c mice which differ in MHC haplotype but both strains are immune to *S. neurona*
induced encephalitis (Royspal et al, 2002). Therefore, it is suggested that there is perhaps a genetic predisposition to *S. neurona* encephalitis and in an immune compromised state this becomes detrimental such as what is observed in immune compromised SCID mice of different genetic backgrounds.

Additionally, author of the current study infected NOD. Cg-*PrkdC*<sup>scid</sup>*Il2rg<sup>tm1wjl</sup>/SzJ (NSG) mice with *S. neurona* merozoites from the SnOP-15 isolate. These mice lack lymphocytes and have dysfunctional natural killer cells which severely limits their ability to fight intracellular infections. Despite these immunological deficits the NSG mice did remain neurologically normal for significantly longer than the B6 *Ifng*<sup>-/-</sup> control mice but eventually succumbed to neurological disease (Figure 1C).

![Figure 2: Representative images of cerebellum samples collected from neurologic mice after *S. neurona* infection where the dark brown staining indicates *S. neurona*. A. Cerebellum collected from B6*scid* mouse. B. Cerebellum collected from B6 *Rag1*<sup>-/-</sup> mouse.](image-url)
Table II: MHC haplotype of investigated mouse models

<table>
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<tr>
<th>Mouse strain</th>
<th>S. neurona Susceptibility</th>
<th>MHC haplotype</th>
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<tr>
<td>Balb/c</td>
<td>No</td>
<td>H2^d</td>
</tr>
<tr>
<td>Balb/c Ifnγ -/-</td>
<td>Yes</td>
<td>H2^d</td>
</tr>
<tr>
<td>Balb/c scid</td>
<td>No</td>
<td>H2^d</td>
</tr>
<tr>
<td>C57Bl/6 (B6)</td>
<td>No</td>
<td>H2^b</td>
</tr>
<tr>
<td>B6 Ifnγ -/-</td>
<td>Yes</td>
<td>H2^b</td>
</tr>
<tr>
<td>B6 nude</td>
<td>Yes</td>
<td>H2^b</td>
</tr>
<tr>
<td>B6 scid</td>
<td>Yes</td>
<td>H2^b</td>
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<tr>
<td>B6 Rag 1 -/-</td>
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</tr>
<tr>
<td>B6eNos -/-</td>
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</tr>
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<td>B6 β2m -/-</td>
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<td>B6 IgH -/-</td>
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</tr>
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<td>H2^g^7</td>
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<td>H2^g^7</td>
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<td>H2^g^7</td>
</tr>
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</table>

### III. The role of T lymphocytes in S. neurona infection

Marsh et al (1997) was the first to highlight the importance of T lymphocytes in the protective immune response against S. neurona infection by demonstrating encephalitis in B6 nude mice. This was further confirmed by Dubey and Lindsay (1998) and later on Witonsky et al (2005a) exemplified the more specific role of CD8+ T-lymphocytes in the protective cell mediated immune response. The B6.129P2- β2m^tm1/unc^ knockout mouse (B6 β2m -/-), which lack CD8+ T lymphocytes succumbed to protozoal encephalitis after infection with 1x10^7 S. neurona merozoites derived from the SnOP-15 isolate (Witonsky...
et al, 2005a). Authors of the current review sought to investigate the role of T-lymphocyte derived IFNγ in fulfillment of neurologic disease, by adoptively transferring splenic T-lymphocytes from wild type C57BL/6 (B6) mice and B6 Ifnγ -/- , into disease susceptible B6 scid mice. After infection with 2x10⁶ S. neurona merozoites derived from the SnOP-15 opossum isolate, neurologic signs occurred on average 25 DPI, in 100% of mice which received B6Ifnγ -/- T-lymphocytes and positive control B6Ifnγ -/- mice. While the group of B6scid mice which received B6 T-lymphocytes remained neurologically normal until the conclusion of the study at 37 DPI (Figure 3A). After histological analysis of cerebellums, S. neurona protozoal encephalitis was confirmed in B6Ifnγ -/- T-lymphocytes and positive control B6Ifnγ -/- mice and both encephalitis and S. neurona were absent in B6 T-lymphocytes recipient mice (Figure 3B). These results imply a need for IFNγ producing T lymphocytes in the protective cell mediated immune response against S. neurona infection in the B6 scid mouse, highlight the importance of both T lymphocytes and IFNγ. The discussed studies establish importance of T-lymphocytes and IFNγ in conferring immunity against S. neurona infection. The role of B lymphocytes in immunopathogenesis has also been investigated.
IV. The role of B lymphocytes in *S. neurona* infection

Activated B lymphocytes generate antibodies against the pathogen responsible for initiating an immune response and subsequent B lymphocyte activation. It is known that both EPM resistant and susceptible horses generate antibodies against *S. neurona* after exposure and to provide insight on whether antibodies are necessary for disease protection or just a consequence of the activated immune response, B-lymphocyte deficient mice (B6.129S2-Igh-6tmlcgn/J) were infected with $1 \times 10^7$ *S. neurona* merozoites from the SnOP-15 isolate (Witonsky et al., 2005b). After as long as 240 days post infection, the infected B lymphocyte deficient mice did not develop neurologic disease. This suggest that antibodies are not an essential component of the protective immune response (Witonsky et al., 2005b). The author of the current review more specifically assessed the importance of the role of antibody affinity and antibody isotype in the immune response against *S. neurona* encephalitis. The NOD/ShiLt-Aicda<sup>em1Cml</sup>

![Figure 3](image-url)

**Figure 3:** A. Neurologic incidence plot with percent neurologically normal mice versus days post *S. neurona* infection. B. Representative IHC image of cerebellum collected from recipient of non-IFNγ producing T lymphocytes. Dark brown staining is *S. neurona*. 
/DvsJ (NOD Aicda -/- ) mouse was infected with $2 \times 10^6 S. neurona$ merozoites from the SnOP-15 isolate. The knockout of the gene Aicda (activation-induced cytidine deaminase) disrupts the processes of class switch recombination and somatic hyper mutation, which are necessary for generation of antibody isotypes other than IgM and antibody affinity maturation, respectively. The NOD Aicda -/- mice were observed for 165 DPI and no neurologic abnormalities presented in these mice nor were any abnormalities observed in NOD mice without the Aicda deletion, but positive control B6 Ifnγ -/- did develop encephalitis (Figure 4). The results of this study confer with Witonsky et al (2005b) that the humoral immune response doesn’t play an essential role in the protective immune response against S. neurona in naïve mouse models with impaired humoral immunity. However, different antibody isotypes may play a role in monitoring disease progression after S. neurona inoculation and/or antiprotozoal treatment as indicated by Hay et al (2019) and therefore the importance of S. neurona antibodies should not be undermined. As previously mentioned, encephalitis can be readily induced in the Balb/c Ifnγ -/- or B6 Ifnγ -/- mice but the precise mechanism(s) that IFNγ acts upon to infer protection is unknown but what has been investigated on this topic is discussed below.
Figure 4: Neurologic incidence plot with percent neurologically normal mice versus days post *S. neurona* infection. *** P <.0001. All comparisons analyzed with Log-rank (Mantel-cox) test.

V. Investigating the role of interferon gamma in *S. neurona* encephalitis

The pleiotropic immune mediator, IFNγ, protects against intracellular infections such as the parasitic pathogen, *S. neurona*. This interferon is produced by both innate immune cells such as natural killer cells and dendritic cells and also the adaptive immune cells, cytotoxic killer T-lymphocytes (CD8+ T-lymphocytes) and by the T\(_H\)1 subset of T-helper lymphocytes. The cytokine increases MHC expression for antigen presentation, increases the cytotoxic nature of CD8+ T-lymphocytes and skews the phenotype of T helper lymphocytes to the pro-inflammatory T\(_H\)1 phenotype, all of which are needed to fight an intracellular infection. To delve further into the role of IFNγ in disease protection investigators infected a variety of mice with genetic mutations relating to IFNγ production or effects. The immune mediator inducible nitric oxide synthetase (iNOS) is induced by cytokines such as IFNγ and constitutively expressed endothelial nitric oxide synthetase (eNOS) function is upregulated by IFNγ. Likewise, it has been reported that
NOS plays a role in the protective immune response against other parasitic infections such as *Toxoplasma gondii* which can lead to cerebral encephalitis in immunocompromised individuals (Schluter et al, 1999). This would suggest that NOS may also play a role in resolving *S. neurona* infection but Rosypal et al (2002), reported that both the C57BL/6 iNOS knockout mouse and C57BL/6 eNOS knockout mouse were resistant to neurologic disease induced by oral inoculation of $4 \times 10^4$ *S. neurona* sporocysts from the Sn-OP15 isolate. The B6 iNOS and eNOS knockout mice were observed for 64 DPI and while the positive control Balb/c *Ifnγ-/-* mice succumbed to *S. neurona* induced encephalitis, both NOS knockout strains of mice did not (Rosypal, 2002). While NOS is IFNγ mediated and offers protection during other intracellular infections it seems that it is not crucial for conferring immunity against *S. neurona* infection and encephalitis. In addition to mediating iNOS function during infection, IFNγ increases the expression of MHC complex and consequently antigen presentation.

The β2m molecule is one of the two main protein chains that compose the MHC I molecule and without β2m MHC I is dysfunctional and consequently a variety of immunological functions are as well. One of those immunological functions is CD8+ T lymphocytes which are predominant producers of IFNγ and depend on MHC I expression for full maturation and are therefore deficient in β2m null mice. As mentioned, Witonsky et al (2005a) investigated disease susceptibility in B6 β2m -/- mice and reported that after infection with *S. neurona* merozoites B6 β2m -/- mice developed protozoal encephalitis. Evolvement of neurologic disease as a result of *S. neurona* infection occurred 150-180 DPI in the β2m -/- mice, which was notably slower than positive control B6 *Ifnγ-/-* mice, who became neurologic by 30 DPI. The results of this study insightfully demonstrate the
necessity for expression of the β2m molecule for complete resolvent of *S. neurona* infection. However, if the interplay of IFNγ and CD8+ T-lymphocytes was the sole component to the protective immune response against *S. neurona* infection, it would be expected that neurologic disease would have occurred at a rate that more closely paralleled the B6 *Ifnγ*-/-positive control mice. Lastly the literature reports that ICR scid mice are not susceptible to *S. neurona* infection and depletion of IFNγ producing natural killer cells does not warrant encephalitis either but when IFNγ is neutralized in vivo the ICR scid mouse succumbs to protozoal encephalitis after infection with *S. neurona* sporocyst (Sellon et al, 2004). In the context of Sellon et al (2004) study, these results indicate that innate immune cells other than natural killer cells can produce enough IFNγ to control *S. neurona* infection. The exact mechanism by which IFNγ acts upon the immune system to generate a protective response and eliminate *S. neurona* infection remains to be fully elucidated. The literature strongly suggests that the interaction of IFNγ, MHC expression and T lymphocyte mediated immunity is involved in the protective immune response, at least in the EPM mouse model.

**Conclusions/Discussion**

While beneficial studies have been conducted over the past decades there remains a gap in the knowledge of the immunopathogenesis of EPM. The results of the studies reviewed here indicated that the IFNγ driven immune response seems be of utmost importance to prevent onset of clinical neurologic disease in *S. neurona* infected mice. Also, study results allude to a strong potential of genetic predisposition since immune compromised mice with certain MHC haplotypes seem to be disease resistant after *S. neurona* infection. Future mouse model studies could include *S. neurona* infection of NOD scid
mice which have the same MHC haplotype as disease resistant ICR scid mice. If disease resistance in the lymphocyte null mouse was dependent on MHC haplotype then presumably the NOD scid would be resistant as well. Also, a B6 scid mouse with the Balb/c MHC haplotype (H2d) could be infected with S. neurona to further investigate the MHC haplotype role in immunopathogenesis. The disease resistant mouse models discussed in the current review display no histological evidence of S. neurona within the CNS or observable neurologic signs. This implies that either the immune response supported elimination of the parasite prior to neural invasion or the immune response within the CNS was able to eliminate the parasite without causing extensive pathological damage. It has been demonstrated in T. gondii mouse models that IFNγ production by microglia cells, resident CNS immune cells, is essential for containment of latent T. gondii infection (Sa et al, 2015). Future studies utilizing mice with specific mutations that effect only IFNγ production in the CNS would help determine if disease protection is a result of the integrated role of peripheral immune response and the CNS immune response or not. There are many different S. neurona isolates which can differ genetically, potentially altering pathogenicity (Asmundsson et al, 2006), and sporocyst viability and merozoite culture passage number can also potentially impact virulence (Cheadle et al, 2001). The studies reviewed here utilized Ifny-/- mice as positive controls to ensure S. neurona virulence but whether these factors impact disease outcome in other immune compromised mouse strains, is unknown and this should be considered in future studies. Likewise, it has been shown that either oral or subcutaneous inoculation of opossum derived sporocysts or subcutaneous inoculation of merozoites results in encephalitis in B6Ifny -/- mice (Dubey et al, 2001). Oral inoculation of sporocysts most
closely resembles infection route in the horse and both sporocysts and merozoite infection results in similar disease outcome in the mouse, but inoculation method should be considered when interoperating results and translating them to the horse. Overall, mouse models have helped narrow the gap in the knowledge of EPM immunopathogenesis, but further studies are still warranted.

References


Schluter, D., Deckert-Schluter, M., Lorenz, E., Meyer, T., Rollinghoff, M., Bogdan, C., 1999. Inhibition of inducible nitric oxide synthetase exacerbates chronic cerebral toxoplasmosis in Toxoxplasma gondii- susceptible C57BL/6 mice but does not reactive the latent disease in T. gondii-resistant BALB/c mice. Journal of Immunology. 162, 3512-3518.


Chapter VI: **Interferon gamma producing T-lymphocytes help prevent Saracocystis neurona induced myeloencephalitis in mouse model**

**Abstract**
Equine protozoal myeloencephalitis (EPM), is predominantly caused by the protozoa *Saracocystis neurona* and is a prevalent neurologic disease affecting horses in North America. Mouse models simulating EPM are commonly used to study the disease due to numerous challenges associated with experimentally inducing EPM in horses. In the current study, the C57Bl/6 scid mouse was found to be susceptible to *S. neurona* encephalitis but the transfer of interferon gamma producing T lymphocytes into B6scid mice offered protection against encephalitis. The transfer of IFNγ deficient T lymphocytes did not offer protection and recipient mice succumbed to disease after infection with *S. neurona*. The results of this study exemplify the need for IFNγ production by T lymphocytes for T lymphocytes to offer protection against *S. neurona* encephalitis.

**Introduction**
Equine Protozoal Myeloencephalitis (EPM), predominantly caused by the protozoa *Saracocystis neurona* (Dubey et al, 1991) is a widespread, neurologic disease of horses in the United States. Horses ingest *S. neurona* sporocyst when feed and water sources become contaminated by opossum feces, which harbor infective life stages of the parasite. The presences of *S. neurona* specific antibodies in equine sera indicates exposure to *S. neurona* and the incidence of exposure is far greater than clinical disease incidence. Sera antibody prevalence and clinical disease development are not directly correlated, and it’s estimated that only 1% of exposed horses develop clinical neurologic disease (Reed et al, 2016). The mechanism defining the immune response which protects
most horses exposed to *S. neurona* from developing neurologic disease remains to be fully elucidated. Investigations have been in both experimentally and naturally affected horses to help define the immunopathogenesis of EPM (Saville et al, 2001, Ellison et al, 2004, Sellon et al, 2004a, Spencer, 2005, Yang et al, 2006, Witonsky et al, 2008, Lewis, 2014). However, it is difficult to use the horses as model to study EPM due to physiological and genetic differences amongst individual horses, ethical concerns and expenses related to using the horse as an experimental and therefore mouse models are more frequently used.

Mouse model studies have highlighted the importance of the cytokine interferon gamma (IFNγ) and the T-lymphocyte mediated immune response for protection against *S. neurona* infection. T-lymphocytes of the Th1 and cytotoxic (CTL) phenotypes are predominant producers of IFNγ, along with innate immune cells such as natural killer cells and macrophages. Initial mouse model studies utilized immune compromised mice to investigate immunopathogenesis in *S. neurona* induced encephalitis in the mouse model. The C57Bl/6 Foxn1<sup>nu</sup> Nu/J (B6 nude) mice which lack T-lymphocytes (Marsh et al, 1997), interferon gamma knockout (Ifnγ<sup>−/−</sup>) mice (Dubey and Lindsay 1998, Dubey 2001, Witonsky et al, 2003a, Hay et al, 2019) and C57Bl/6, β2m knockout mice which lack MHC I expression and consequently, functional CD8+ T-lymphocytes (Witonsky et al, 2005a) were utilized to demonstrate the importance of T lymphocytes and cytokine IFNγ in protection against *S. neurona* induced encephalitis. Upon infection with *S. neurona* all previously mentioned mouse strains demonstrate pathological evidence of *S. neurona* induced encephalitis and apparent neurologic signs. Mice with T-lymphocytes but lacking functional B-lymphocytes (mµMT<sup>−/−</sup>) are disease resistant (Witonsky et al,
Interestingly, discrepancies exist in previous reports of the incidence of neurologic disease in severe combined immunodeficiency (SCID) mice. The SCID mice are lymphocyte deficient due to the protein kinase DNA activated catalytic polypeptide (Prkdc) mutation, which disrupts the maturation processes in lymphocytes. The SCID mouse has an intact and functional innate immune system and produces some IFN\(\gamma\) but lack functional T and B lymphocytes. Therefore, the potential protective role of T-lymphocytes and associated IFN\(\gamma\) production is also lacking in the SCID mouse. Marsh et al (1997) reported that ICR-\(\text{Prkdc}^{\text{scid}}\) (ICR scid) mice do not develop neurologic disease associated with \(S.\) \(nuerona\) infection but Ahlgrim et al (unpublished work) reported that disease occurs in B6. CB17-\(\text{Prkdc}^{\text{scid}}/\text{SzJ}\) (B6 scid) and CB\(\text{BySmn}\). CB17 - \(\text{Prkdc}^{\text{scid}}/\text{J}\) (Balb/c \(\text{scid}\)) are disease resistant. These discrepancies potentially relate to genetic differences in the different mouse strains and IFN\(\gamma\) production as splenocytes collected from Balb/c mice have been reported to have increased IFN\(\gamma\) production \textit{in-vitro} (Koo et al, 2006). Additionally, Sellon et al (2004b) reported that depletion of IFN\(\gamma\) results in neurologic disease in \(S.\) \(neurona\) infected ICR \(\text{scid}\) mice further supporting the role of this cytokine in disease prevention.

These conflicting results suggest that SCID mice may be susceptible to disease under certain circumstances and therefore further investigation is warranted as characterizing these differences may yield valuable information. In the current study we determined the neurologic disease susceptibility related to \(S.\) \(neurona\) infection in lymphocyte null B6\(\text{scid}\) and Balb/c \(\text{scid}\) mice. Furthermore, we sought out to further define the role of T-lymphocytes and T-lymphocyte derived IFN\(\gamma\) in protection against \(S.\)
neurona induced encephalitis through adoptive transfer of IFNγ competent of deficient T-lymphocytes.

Materials and Methods
Mice
Male mice age 8-10 weeks old of the following strains; B6. 129S7- Ifngtm1Ts/J (B6. Ifnγ -/-) (JR 2287), B6.CB17-Prkdc scid /SzJ (B6. scid) (JR1913), CBBySmn.CB17-Prkdc scid /J (Balb/c scid) (JR1803) and C57Bl/6 (B6) (JR 664) were obtained from Jackson Laboratory (Bar Harbor, Maine). The B6. Ifnγ -/- mice were maintained in accordance with an Institutional Animal Care and Use Committee (IACUC) breeding protocol and for these mice and all others mentioned, experimental studies were conducted under other IACUC approved protocols.

For the neurologic disease susceptibility experiments in lymphocyte null mice, Balb/c scid (n=10) and B6. scid (n=10) mice (Table I), were infected with 2 x 10^6 S. neurona merozoites via subcutaneous injection on Day 0. Blood was collected via submandibular venipuncture from a cohort of both Balb/c. scid (n=5) and B6. scid (n=5) mice on Day 0 before S. neurona infection and then on a weekly basis for 3 weeks; 7 days post infection (DPI), 14 DPI and 21 DPI and upon the development of neurologic signs or 39 DPI when mice were euthanized and blood was collected post euthanasia via cardiac puncture. The mice used for weekly blood samples were ear notched for identification. Blood was collected from the remainder of the mice post euthanasia which occurred upon the development of neurologic signs or 60 DPI via cardiac puncture (B6. scid n=5) or (Balb/c scid n=5). Sera was collected after centrifugation and stored at -20 C for further analysis. B6. Ifnγ -/- mice (n=5) were used as positive controls to test S. neurona virulence. All mice were euthanized using CO₂ asphyxiation and development
of neurologic disease was characterized by clinical neurological signs relating to lack of balance and coordination. At the time of euthanasia cerebellum samples were collected for histological analysis.

For the T-lymphocyte transfer experiments the B6 and B6 Ifnγ -/- mice intended for the use as T-lymphocyte donors, were euthanized and spleens were collected for cell isolation. For the transfer experiments 3x10^6 T-lymphocytes were intraperitoneally injected into B6. scid recipient mice (n=10). The mice were divided into two recipient groups; those which received IFNγ producing T-lymphocytes, T_IFNγ (n=3) and those which received IFNγ deficient T-lymphocytes, T_IFNγ -/- (n=7) (Table II). Blood was collected via submandibular venipuncture from all these mice 3 weeks post T-lymphocyte transfer for analysis of circulating T-lymphocytes via flow cytometry. At 6 weeks post cell transfer mice were infected with 2x10^6 S. neurona merozoites via subcutaneous injection. All mice were euthanized using CO₂ asphyxiation when clinical neurological signs relating to lack of balance and coordination were observed or at 37 DPI. Post euthanasia blood was collected via cardiac puncture for flow cytometry analysis of circulating T-lymphocytes and sera analysis for IFNγ, spleens were collected for further immune cell analysis via flow cytometry and cerebellum samples were collected for histological analysis.

Saracocystis neurona culture and preparation of inoculum

Merozoites of the sixth passage of the SN-15OP isolate were grown in African green monkey (Cercopithecus aethiops) kidney cells (CV-1, ATTC CCL-70, Manassas, VA, USA), separated from host cells, collected and enumerated for inoculations as previously described (Lindsay et al, 2013). The SN-15OP isolate was isolated from the brain of an
Balb/c Ifnγ−/− mouse fed sporocysts obtained from the feces of a naturally infected opossum from Virginia (Dubey, 2000). This isolate was originally a gift from Dr. J. P. Dubey, United States Department of Agriculture, Beltsville, Maryland USA and has been maintained cryopreserved in liquid nitrogen.

Table I: Neurologic disease susceptibility

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Weekly Blood samples</th>
<th>Neurologic Signs</th>
<th>IHC Positive</th>
<th>Encephalitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6. Scid n=10</td>
<td>Yes 5/10</td>
<td>Yes 10/10</td>
<td>Positive 5/5</td>
<td>Yes 5/5</td>
</tr>
<tr>
<td>Bab/lc. scid n=10</td>
<td>Yes 5/10</td>
<td>Yes 1/10a</td>
<td>Positive 1/5b</td>
<td>Yes 1/5c</td>
</tr>
<tr>
<td>B6 Ifnγ -/−</td>
<td>No 5/5</td>
<td>Yes 2/2</td>
<td>Positive 2/2</td>
<td>Yes 2/2</td>
</tr>
</tbody>
</table>

a All other mice (n=8) were euthanized at 39 DPI (n=4) or 60 DPI (n=5)
b All other mice (n=4) were negative for S. neurona staining
c All other mice (n=4) did not show signs of encephalitis

Cerebellum histological analysis

For the neurologic disease susceptibility trial there were five cerebellar samples collected post euthanasia from each the Balb/c scid and B6scid groups and two from the positive control B6Ifnγ−/− mice (Table I). For the T-lymphocyte transfer trial all cerebellums were collected from all transfer recipient mice (Table II). All collected cerebellums were fixed in 10% formalin, embedded in paraffin sectioned for hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. The H&E stained samples were assessed for total number of perivascular cuffs, gliosis foci and IHC for number of S. neurona organisms. For each assessed parameter a score was assigned 1 (nothing present) to 8 (greatest number of cuffs, foci or organism). Sections of cerebellum were stained with polyclonal
rabbit anti-\textit{S. neurona} anti-sera as described by Gerhold et al. (2005) with the exception that sections were digested with peroxidase suppressor (Thermo Fisher, Waltham, Massachusetts) prior to incubation with anti-sera at 1:500 dilution.

\textbf{Table II: T-Lymphocyte Transfer Experiment}

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Donor or Recipient</th>
<th>Neurologic Signs</th>
<th>IHC</th>
<th>Encephalitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>Donor n=5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B6 Ifn γ -/-</td>
<td>Donor n=5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>TIFNγ\textsuperscript{a}</td>
<td>Recipient n=7</td>
<td>Yes</td>
<td>Positive 7/7</td>
<td>Yes 7/7</td>
</tr>
<tr>
<td>TIFNγ\textsuperscript{b}</td>
<td>Recipient n=3</td>
<td>No</td>
<td>Positive 0/3</td>
<td>Yes 0/3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mice received T-lymphocytes isolated from B6 Ifn γ -/-

\textsuperscript{b}Mice received T lymphocytes isolated from B6 mice

\textbf{T-lymphocyte isolation}

Spleens collected for T-lymphocyte transfer experiments were homogenized into a single cell suspension and treated with ACK lysis buffer. Splenocytes were incubated with anti-Mac1- biotin (M1/70)(BD Biosciences San Jose, CA) and anti-B220-biotin (RA3-62B)( BD Biosciences San Jose, CA) for 30 mins at 4 C, washed with 1x PBS, then incubated with streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 mins 4 C and then passed through MACS LD columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufactures protocol. The enriched splenocytes were resuspended at 1.5×10\textsuperscript{7} ml\textsuperscript{-1} in sterile 1x PBS and 3×10\textsuperscript{6} T-lymphocytes were transferred to each recipient mouse via intraperitoneal injection.

\textbf{Flow cytometry}
For the T-lymphocyte transfer experiments all blood samples collected were treated with sodium EDTA directly after collection. The collected spleen samples were homogenized into a single cell suspension. Both blood and spleen samples were then treated with ACK lysis buffer and then cells were washed twice in 1x PBS twice. Both peripheral blood leukocytes (PBL) and splenocytes were counted on Nexcelom cell counter and resuspended at 2x 10^7 cells/mL. Cells were stained with fluorochrome conjugated mouse antibodies against CD3 (145-2C11), CD4(GK15), CD8(53-6.7) and B220(RA3-6B2) (Biolegend, SanDiego, CA) and incubated at 4 C for 30 mins. Directly before conducting flow cytometry propidium iodide (PI) viability dye was added to each sample to differentiate between live and dead cells. All samples were run on Attune NxT cytometer (Thermo Fisher Scientific) with Attune NxT software v 2.4 (Life Technologies, Thermo Fisher, Waltham, MA). Live Cells were gated for single cell populations and then for lymphocytes using FSC vs SSC. All data analysis was completed utilizing FlowJo software (FlowJo, LLC, Ashland, OR).

**Serum Interferon gamma ELISA**

Sera IFN\(\gamma\) concentrations were measured using standardized ELISA kit, Mouse IFN\(\gamma\) ELISA kit (Invitrogen, ThermoFisher, Ref # 88-7314). The manufacturers protocol was followed. Plates were read on an Infinite M200 Pro plate reader using Magellan 7.0 software (Tecan).

**Statistical Analysis**

For determining significance in the neurologic incidence data, a log-rank Mantel-Cox test with a significant level set at P < 0.05 and Bonferroni correction to account for multiple comparisons with a significance threshold at P <0.017. Significance for measured
histological parameters was determined using One-way ANOVA and Tukey’s T-test for multiple comparisons for the neurologic disease susceptibility trial and Mann Whitney T-test for the T lymphocyte transfer experiment. Two-way ANOVA and Tukey Test for multiple comparisons was used to determine significance for the flow cytometry data and ELISA data. The significance level for all analysis was set at $P < 0.05$. All analyses were performed using GraphPad Prism software version 6 for Mac (GraphPad Software, La Jolla, California).

**Results**

**Neurologic Disease Incidence**

![Graph showing neurologic incidence](image)

**Figure 1.** A and B. The percent of neurologically normal mice vs. Days post *S. neurona* infection. The time point Day 0 represents the day of infection. Closed circles represent the number of neurologically normal mice at that time point. **** $P \leq 0.0001$, *** $P \leq 0.0005$ and ** $P \leq 0.005$.

The incidence of neurologic disease occurred at a significantly greater rate in the B6. *scid* mice than the Balb/c. *scid* mice with neurologic incidence rate of 100% in the B6. *scid* mice and a rate of only 10% in the Balb/c. *scid* mice with in 60 DPI (Figure 1A). On average the B6. *scid* mice exhibited neurologic signs at 35 DPI and the one Balb/c *scid*
mouse demonstrated signs at 35 DPI. All positive control \( Ifn\gamma \)-/- mice developed neurologic signs within 30 DPI (Figure 1A). The onset of neurologic signs relating to lack of balance and coordination did occur in both strains of SCID mice but at a significantly lower rate in the Balb/c scid mice, suggesting that these mice do mount an immune response against \( S. neurona \) which is more protective than the response of the B6 scid. Based on these results the B6. scid mouse was selected as the recipient mouse for the T-lymphocyte transfer experiments conducted in the current study.

For the T-lymphocyte transfer experiment, there was a 100% neurologic incidence in the group of mice that received IFN\( \gamma \) deficient T-lymphocytes (T_{IFN\gamma/-}) with an average rate of 25 DPI. All positive control \( Ifn\gamma \)-/- mice displayed neurologic signs within 30 DPI. The transfer of the IFN\( \gamma \) producing T-lymphocytes (T_{IFN\gamma}) decreased the rate of neurologic incidence, as all mice in this group did not develop neurologic signs by the conclusion of the observation period at 37 DPI. The study was concluded at 37DPI because this time period exceeded the average mortality rate (26 DPI) of B6 \( Ifn\gamma \)-/- positive controls. These results indicate that the transfer of IFN\( \gamma \) producing T-lymphocytes positively influenced the rate of neurologic incidence by decreasing the development of neurologic signs. However, the transfer of the IFN\( \gamma \) deficient T-lymphocytes did not (Figure 1B).

**Histopathological analysis**

For both the neurologic disease susceptibility (Table I) and T-lymphocyte transfer (Table II) experiments the histology results confirmed \( S. neurona \) induced encephalitis in all mice that displayed clinical neurologic signs and those that did not display clinical signs
did not develop encephalitis. *Sarcocystis neurona* induced encephalitis was characterized in the collected cerebellar samples by the presences of both gliosis and perivascular cuffing and the finding of *S. neurona* by IHC staining. For the neurologic disease susceptibility trial there was significantly more perivascular cuffing present in the B6 scid mice than Balb/c scid mice and the B6. *Ifnγ/-* had significantly greater amounts of perivascular cuffing than both groups of SCID mice (Figure 3A). There was a significantly greater amount of gliosis foci in the B6scid cerebellum samples compared to the Balb/c scid and the positive control cohort, B6 *Ifnγ/-*, had significantly greater number of gliosis foci than both B6 scid and Balb/c scid mice (Figure 3B). *Saracocystis neurona* was only found in one of the cerebellum samples collected from the Balb/c scid mice and therefore there was significantly less *S. neurona* in this group compared to the B6 scid and B6 *Ifnγ/-* mice. The B6 *Ifnγ/-* mice had largest number of *S. neurona* and significantly more than mice in both SCID groups (Figure 3C).

The transfer of IFNγ producing T-lymphocytes did not significantly alter the presences of perivascular cuffing between the two recipient groups (Figure 3D) and there was an insignificant increase in the T<sub>IFNγ/-</sub> group compared to the T<sub>IFNγ</sub>. There were no gliosis foci identified in the T<sub>IFNγ</sub> group and therefore there was significantly greater number of gliosis foci in the T<sub>IFNγ/-</sub> group (Figure 3E). Likewise, no *S. neurona* was identified by IHC in the T<sub>IFNγ</sub> group and therefore there was significantly greater amount of positive *S. neurona* IHC staining in the T<sub>IFNγ/-</sub> group (Figure 3F). The transfer of IFNγ producing T lymphocytes resulted in improved pathology and no encephalitis compared to the transfer
of IFNγ deficient T lymphocytes highlighting the importance of the cytokine IFNγ not just T-lymphocytes.

**Figure 3.** Scores of the measured histological changes associated with *S. neurona* infection for both the neurologic disease susceptibility trial (A-C) and the T-lymphocyte transfer trial (D-F). A,D. Perivascular cuffing foci in H&E stained cerebellum sections. B,E. Gliosis Foci in H&E stained cerebellum sections. C,F. *Sarcocystis neurona* organisms in IHC stained cerebellum sections. Data is present as ± SEM. * P ≤ 0.05, ** P ≤ 0.005, *** P ≤ 0.0005, **** P ≤ 0.0001.
Flow Cytometry

The peripheral blood samples collected at 3 weeks post T-lymphocyte transfer exhibited CD3+ T-lymphocytes in circulation for both recipient groups with no significant differences between the two groups (data not shown), confirming successful T-lymphocyte transfer. Splenocytes and PBL samples collected from transfer mice post euthanasia were analyzed for populations of CD4+ and CD8+ T-lymphocytes. In the T$_{IFN\gamma}$ group there was a trending increase ($p = 0.05$) in CD8+ T-lymphocytes in the spleen compared to the T$_{IFN\gamma}^{-/-}$ group but no differences in the CD4+ T-lymphocyte population (Figure 4A). The results from the PBL samples demonstrate a slight but insignificant increase in both CD4+ and CD8 + T lymphocytes in circulation in the T$_{IFN\gamma}^{-/-}$ group compared to the T$_{IFN\gamma}$ group (Figure 4B). The increase in T-lymphocyte populations in circulation in the T$_{IFN\gamma}^{-/-}$ may be due to persistent S. neurona infection and a resolution of infection in the T$_{IFN\gamma}$ group.

**Figure 4.** Flow cytometry data analyzing differences in CD4+ T lymphocyte and CD8 + T lymphocyte populations in splenic (A) and peripheral blood leukocytes (B). Spleen and blood samples were collected at the time of neurologic sign development for TIFN$\gamma$ -/- group and 37 DPI for the TIFN$\gamma$ group. No significant differences occurred but there was a trending increase ($P= 0.05$) in splenic CD8+ T-lymphocyte population (A). Data is presented as $\pm$ SEM.
Interferon gamma Enzyme linked immunosorbent assay (ELISA)

In the neurologic disease susceptibility experiment when comparing sera IFNγ concentration at day 0, 7DPI, 14 DPI and 21 DPI for mice in both the B6 scid (n=5) and Balb/c scid (n=5) groups IFNγ remained below the detection limit of the kit, until day 21 DPI. At 21 DPI, 2 of 5 B6 scid mice had an increase in sera IFNγ concentration but Balb/c scid mice remained below detection limit. Upon the development of neurologic signs in the B6 scid group the average sera concentration of IFNγ was 1376 pg/mL but remained below detection level in the Balb/c scid mice at the time of euthanasia. These results indicate that as infection persisted in the B6 scid mice serum IFNγ concentrations increased but mice still developed neurologic disease. Conversely, in Balb/c scid mice IFNγ concentrations remained below detection limit of the ELISA suggesting that S. neurona infection did not result in a significant increase in IFNγ production these mice (Figure 5). Serum sample concentrations were altered to improve IFNγ detection but results mostly remained below detection limit.

Discussion
Conversely to previous investigations our results from this study indicate that when B6scid and Balb/c scid mice are infected with opossum derived SN-15OP S. neurona merozoites both strains of mice develop neurologic disease but at altered rates. The B6scid mice demonstrated fulminant neurologic disease with all mice demonstrating neurologic signs on average 35 DPI and cerebellar histology associated with S. neurona encephalitis. The B6scid mice developed neurologic signs at a significantly slower rate than positive control B6Ifnγ-/- mice, which on average developed signs 23 DPI. Given that B6scid mice still have a functional innate immune system and therefore some IFNγ production it is likely that this helped control S. neurona infection and prolong development of neurologic signs but was evidently not enough to entirely prevent disease. The Balb/c scid mice were nearly disease resistant with only one mouse demonstrating neurologic signs at 35 DPI and cerebellar histology associated with S. neurona infection. The remaining Balb/c scid mice remained neurologically normal throughout the study and presented with normal cerebellar pathology at 39 DPI or 60 DPI. Based on results from previous investigations we hypothesized that if disease susceptibility differed between the B6 scid and Balb/c scid strains this was potentially due to an increase production of innate immune cell derived IFNγ in response to S. neurona infection. Sellon et al (2004) demonstrated that ICR scid mice are resistant to disease when infected with S. neurona but when IFNγ was depleted in ICR scid mice they developed neurologic disease. Koo et al (2006) demonstrated that there is a greater production of predominantly natural killer cell derived IFNγ when Balb/c splenocytes were cultured with Burkholderia pseudomallei compared to B6 splenocytes. However, we were not able to detect an increase in circulating IFNγ concentrations in sera samples.
collected weekly for three weeks post *S. neurona* infection in the Balb/c scid mice compared to the B6scid mice. It is possible that a peak of IFNγ production occurred less than 7DPI and the optimal sampling time point to distinguish significant differences between the two groups was overlooked. Another possibility is that the differences in IFNγ production were in the central nervous system influencing neurovirulence which is the case in *Toxoplasma gondii* infection. Sa et al (2015) demonstrated the importance of microglia derived IFNγ in controlling *T. gondii* growth and infection in Balb/c mice. It is also possible that the differences in disease resistance is irrelevant to IFNγ production and may be related to other genetic differences that exist between the two strains of mice. Genetic differences may also explain discrepancies in previous reports as both Marsh et al (1997) and Sellon et al (2004) conducted their studies with ICR scid mice and not Balb/c scid or B6 scid. In the current study we demonstrated that there is a significant difference in neurologic disease susceptibility related to *S. neurona* infection in Balb/c scid and B6 scid mouse strains but the immune response protecting Balb/c scid mice needs to be further investigated. While the current study was conducted in mice, it should be mentioned that *S. neurona* infection and disease susceptibility studies have been conducted in SCID equine foals.

When equine foals with the scid mutation were inoculated with *S. neurona* sporocysts developed parasitemia and infection in visceral tissues (liver, spleen, skeletal muscle, cardiac muscle, kidney, tongue and heart) but not clinical neurologic disease. Immunocompetent control horses (4 of 6 horses) in this study did not demonstrate parasitemia or infection of visceral tissues but did develop neurologic signs relating to *S. neurona* infection by the end of the study period (Sellon et al, 2004). In the mentioned
study two of three SCID foals had to be euthanized 21-32 days post infection due to illness unrelated to S. neurona infection resulting in small sample population. Authors of the current study feel that if this study included more foals and a healthier a health status could be maintained for a longer timeframe than SCID foals may succumb to disease relating to S. neurona infection.

To further investigate the role of T-lymphocyte derived IFNγ in protection against S. neurona induced encephalitis we selected the B6scid mice as adoptive transfer recipients due to their increased disease susceptibility compared to Balb/c scid mice. We found that the transfer of IFNγ producing T-lymphocytes exerted a protective effect on neurologic disease after infection with S. neurona. The recipient group of IFNγ producing T-lymphocytes did not exhibit neurologic abnormalities or characteristic cerebellar lesions associated with S. neurona infection. However, 100% of recipients of Ifnγ−/− T-lymphocytes demonstrated neurologic signs, pathological cerebellar lesions and S. neurona was identified by immunohistochemistry like B6scid mice. Results from the flow cytometry data collected in the current study demonstrate a significant increase in splenic CD8+ T-lymphocytes from T IFNγ compared to the T IFNγ−/− but not CD4+ T-lymphocytes. Previous studies have revealed the importance of CD8+ T-lymphocytes in the protection against S. neurona induced encephalitis as this population of cells increases after infection in immunocompetent C57Bl/6 mice (Witonsky, 2003b) and without functional CD8+ T-lymphocytes mice are susceptible to S. neurona induced myeloencephalitis (Witonsky et al, 2005a). While there was a significant increase in splenic CD8+ T lymphocytes there was not a significant difference in splenic CD4+ T lymphocyte population between the two groups. This suggest and supports previous
reports that CD8+ T-lymphocytes play a more essential role in the protective immune response compared to CD4+ T-lymphocytes in *S. neurona* infection. Cell mediated immune response specifically CD8+ T-lymphocytes and IFNγ is important for protection against other equine pathogens such as equine herpesvirus-1 (Paillot et al, 2005) and other intracellular protozoan infections such as *T. gondii* (Gigley, 2011). The results from the current study exemplifies the need for T-lymphocytes to be capable of producing IFNγ in order to be protective, and suggest a potential role of IFNγ deficient T-lymphocytes in accelerating disease progression because the average rate of neurologic sign development in the T_{IFNγ/-} group was considerably earlier than B6 scid mice. While both B6scid and T_{IFNγ/-} groups of mice were infected with the same strain of *S. neurona* and virulence was confirmed with positive control B6Ifnγ/- mice, the infection time points of these two groups of mice occurred at different timepoints and therefore to make direct comparisons, further investigation is warranted. Collectively these results indicate the importance of IFNγ producing T-lymphocytes in protection against disease development but also that dysfunctional T-lymphocytes may play an essential role for disease development. While this study was conducted with murine models it is likely that if an immunomodulator that increase T-lymphocyte IFNγ production in horses could be identified this would greatly improve prognosis for EPM affected patients. Studies investigating the immunological properties of available immunomodulators for horses have been conducted (Horohov, 2008, and Witonsky, 2019) but their effects on *S. neurona* infection remain to be investigated and further investigation is warranted.

**References**


Chapter VI: *Exploration of methods for investigating the immunophenotype of EPM affected horses*

**Abstract**

Equine protozoal myeloencephalitis (EPM) predominantly caused by the protozoon *Saracocystis neurona*, is a common neurologic disease in horses from North America. Exposure to the parasite occurs frequently but clinical neurologic disease emerges in a small fraction of exposed horses. Histological evaluation of spinal cord samples collected from EPM affected horses has indicated that the immune response following *S. neurona* neuroinvasion is of inflammatory nature, subsequently resulting in CNS damage and clinical neurologic deficits. It is unknown whether an aberrant immune response in the CNS is responsible for neurologic disease or if disease is due to failure of the peripheral immune response to control infection or the fault of both the CNS and peripheral immune response. The goal of this study was to explore histological methods which could be used to characterize an EPM immune phenotype. Characterizing the EPM immune phenotype which could further be used to decipher the immune response to *S. neurona* infection.

The results of this study include the use of in-situ hybridization technique RNAscope for identification of *S. neurona* in spinal cord tissue samples collected from EPM affected horses, proposing a new method for immunopathology analysis for EPM.

**Introduction**

Horses are predisposed to ingestion of pathogens due to environmental contamination, such as the protozoon *Saracocystis neurona* carried in opossum (*Didelphis virginiana*) feces (Dubey and Lindsay, 1998, Dubey et al. 2000, Dubey, 2000). Many horses encounter *S. neurona* and exposure merely results in antibody production against the parasite and no adverse disease (Reed et al, 2016). However, for a select population of horses’ exposure(s) to *S. neurona*, results in the development of clinical neurologic
disease known as equine protozoal myeloencephalitis (EPM). EPM is the most common neurologic disease that affects approximately 14 of every 10,000 horses older than 6 months of age in the United States (NAHMS, 2001).

Histological evaluation of the spinal cord samples collected from EPM affected horses has revealed inflammatory lesions which result in CNS damage and neurologic deficits (Dubey, 2001 and Dubey, 2015). Focal or multifocal pathological lesions can occur anywhere within the CNS of the horse, but typically are found in the cervical and lumbar regions of the spinal cord and the brainstem in the brain. The nonspecific origin of pathological lesions means neurologic signs correlated with EPM range vastly, from mild stumbling to proprioception loss to complete recumbency in more severe cases. The multitude of neurologic signs makes the disease difficult to clinically differentiate from other neurologic diseases and clinicians rely heavily on antibody titers and response to treatment for antemortem diagnosis. Additional biomarkers which could be used to differentiate EPM from other neurologic diseases would improve diagnostic capabilities, but this is limited partly by the knowledge of the immunopathogenesis of EPM. It is not known whether an aberrant immune response in the CNS is responsible for neurologic disease in susceptible horses or if disease is due to failure of the peripheral immune response to control the infection allowing for CNS invasion, or a fault of both the neural and peripheral immune responses. Mouse model studies simulating EPM have demonstrated the necessity of the cytokine IFNγ for disease protection and suggest an importance of pro-inflammatory cell mediated immune response (Marsh et al, 1997 and Witonsky et al, 2005). Altered cell mediated immune response in naturally affected EPM horses and in experimentally infected horses have also been reported (Scott et al, 2005,
Yang et al, 2006, and Witonsky et al, 2008). In the current study authors sought to better define the T lymphocyte mediated immune response in EPM affected horses by in-situ analysis of cytokines correlated with the $T_H^1$ cell response (IFNγ), $T_H^2$ response (IL-4), $T_H^{17}$ response (IL-17) and $T_{reg}$ response (IL-10). Additional histological analysis methods, serologically and CSF analysis methods were explored to attempt to define an EPM immune phenotype, which could further be used to decipher the protective immune response against $S. neurona$ infection.

**Materials and Methods**

**Horses**

A mixed breed population of horses with the average age of 15 years ($\pm$6.8 years), both geldings and mares were used for the current study. The horses were part of either a control group (n=9) or EPM affected group (n=9). The control group consisted of non-neurologic horses with no current history of inflammatory disease or recent administration of anti-inflammatory medication, with serum: cerebral spinal fluid (CSF) $S. neurona$ antibody ratio $>100$ based on SnSAG 2, 3, 4 ELISA testing (Equine Diagnostic Solutions, Lexington, KY), post-mortem exam revealed normal brainstem, cerebrum, spinal cord histology. The EPM affected group consisted of horses with neurologic abnormalities which were assessed utilizing the Mayhew neurologic scale, serum: cerebral spinal fluid (CSF) $S. neurona$ antibody ratio $\leq 100$ based SnSAG 2, 3, 4 ELISA testing (Equine Diagnostic Solutions, Lexington, KY), post-mortem exam revealed pathological lesions consistent with EPM, in the brainstem, cerebrum or spinal cord. For the EPM affected horses, treatment status and duration of clinical signs was noted for consideration during analysis. All horses were admitted to Virginia Maryland
College of Veterinary Medicine (Blacksburg, VA) where collection of blood, cerebral spinal fluid and euthanasia and necropsy occurred at the horse owner’s discretion. Horses were euthanized by intravenous injection of phenobarbital.

**Sample Collection: Spinal cord, Cerebral spinal fluid, Serum**

Blood was collected from each horse by jugular venipuncture prior to euthanasia and sera was collected after centrifugation. Cerebral spinal fluid (CSF) was collected immediately after euthanasia from the atlanto-occipital joint space. Serum and CSF were analyzed by Equine Diagnostic Solutions (Lexington, Kentucky) for *S. neurona* antibodies utilizing the SnSAG 2, 3,4 ELISA. Additionally, serum and CSF samples were analyzed for IFN α, IFNγ, IL-4, IL-10 and IL-17 utilizing the commercially available 5-plex cytokine assay at Cornell University, Dr. Bettina Wagner’s laboratory. The serum and CSF samples were also analyzed by Dr. Wagner’s laboratory for further chemokine/cytokine analysis utilizing a recently developed multiplex assay that measures; IL-1β, TNFα, CCL2, CCL3, CCL11 and CCL 5 and one final assay measuring solubleCD14. The antibodies utilized in these assays are described by Schnabel et al (2018) and Wagner and Freer (2009).

The brain and entire spinal cord were removed from the horse during necropsy by a board-certified pathologist and sections of the spinal cord were collected. For all horses in both the control and EPM groups, sections of the cerebrum and brainstem were collected along with sections of the cervical (C1-7), lumbar (L2-6) and sacral (S1-2) regions of the spinal cord. For the EPM affected horses if clinical neurologic signs appeared to potentially involve the thoracic region then samples were also collected from here as well. The collected tissue samples were placed in optimal compounding tissue (OCT) media and slowly frozen on dry ice, to prevent cracking of samples during
freezing and stored at -80 C for future analysis. Additionally, approximately 3 mm tissue samples were collected and snap frozen on liquid nitrogen for future DNA and RNA extraction. The remainder of the spinal cord was fixed in 10% buffered formalin and tissue adjacent to those collected for frozen tissue analysis was collected for histological analysis.

**Histological analysis**

The formalin fixed sections were paraffin embedded and section and stained with hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. The H&E stained sections were reviewed by a veterinarian board certified pathologist for histological changes related to EPM which included; inflammatory changes (lymphocytic infiltration, eosinophilic inflammation, gliosis, perivascular cuffing), degenerative changes and *S. neurona* itself. For IHC analysis replicate sections to those that the EPM lesion was identified in were stained with rabbit anti-*S. neurona* sera as described by Gerhold et al (2005) with the exception that the anti-*S. neurona* antibody was utilized at a concentration of 1:500 and peroxidase inhibitor (ThermoFisher Scientific) was used for inhibition of endogenous peroxidases. The collected samples from the control horses were also reviewed by a board-certified pathologist to confirm normal CNS pathology and sections were also stained with rabbit anti- *S. neurona* sera to serve as experimental negative controls.

**Immunofluorescent slide staining**

The OCT frozen tissue samples collected from the region where pathological lesions were identified in H&E stained samples, were sectioned on a cryostat in 10µm sections and stained with one of the combinations listed in table I, in order to visualize cytokine
and cell localization. The antibodies used to identify astrocytes were tight junction protein, connexin 43 (Cx43-PE), Glial fibrillary acidic protein (GFAP-FitC) and the antibody for microglia cells ionized calcium binding adaptor molecule 1 (Iba-1-FitC), all antibodies were purchased from SantaCruz BioTech, (Dallas, TX) and used a working concentration of 1:100. The T-lymphocyte antibodies CD4-FitC (concentration 1:100) and CD8-PE (concentration 1:250) were purchased from Bio-Rad (Hercules, CA). The cytokine antibodies (IFNγ, IL-17a, IL-10 and IL-4) were all conjugated with AF-647 and were purchased from Dr. Bettina Wagner Laboratory (Cornell University, Ithaca, NY). Briefly, the staining protocol utilized is as follows, slides were incubated in histological grade acetone 10 mins, 4 C, washed 3x in 1x PBS, tissue sections were encircled with ImmunoPap pen, 3% FBS blocking buffer was added to each section, incubated for 1 hr, room temperature (RT) in a moist chamber, washed 3x in 1 x PBS, incubated with the appropriately diluted antibodies 1 hr RT in a moist chamber for T-lymphocyte markers and cytokines or overnight at 4 C for microglia and astrocyte +cytokines. Following antibody incubation, slides were washed 3x with 1x PBS, stained with nuclear counterstain DAPI for 10 mins RT, washed 3x with 1x PBS and cover slipped and left to dry at RT and stored at 4 C till imaged. Each spinal cord section (n=12 sections/horse) was imaged using an TiE- Nikon fluorescent light microscope utilizing the same light exposure sections for imaging consistency within 48 hours of staining. All sections were scanned for cytokine (IFNγ, IL-17a, IL-4 or IL-10) staining and 4-5, 20x HPF images of cytokine staining were captured to represent the cytokine staining pattern of a particular section. The total number of cytokines in each image was then manually enumerated along with Microglia cells and T-lymphocytes when present in the captured image.
Astrocytes could not accurately be manually enumerated due to the complexity of the cells and therefore fluorescent intensity of the GFAP marker was measured which has previously been described by (Lemos, 2008). A scoring system of 0 (no cells present) to 8 (the greatest number of cells present) was utilized for quantification analysis of cell types in each captured image.

Table I: Antibody combinations for immunofluorescent staining of CNS tissue samples

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Type Antibody</th>
<th>Antibody combinations: Cell Type + Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytes</td>
<td>Cx43</td>
<td>+ IFNγ + IL17a + IL4 + IL10</td>
</tr>
<tr>
<td></td>
<td>+ GFAP</td>
<td>+ Astrocyte + Astrocyte + Astrocyte + Astrocyte</td>
</tr>
<tr>
<td>Microglia</td>
<td>Iba-1</td>
<td>+ IFNγ + IL17a + IL4 + IL10</td>
</tr>
<tr>
<td></td>
<td>Microglia</td>
<td>+ Microglia + Microglia + Microglia + Microglia</td>
</tr>
<tr>
<td>T-lymphocytes</td>
<td>CD4</td>
<td>+ IFNγ + IL17a + IL4 + IL10</td>
</tr>
<tr>
<td></td>
<td>+ CD8</td>
<td>+ T-lymphocytes T-lymphocytes T-lymphocytes T-lymphocytes</td>
</tr>
</tbody>
</table>

*a Overnight incubation period at 4 °C
*b One-hour incubation period at room temperature

**RNAscope**
The In-situ hybridization technique, RNAscope, was utilized to investigate another method to detect *S. neurona* in the CNS of EPM affected horses. The assay allows visualization and quantification of gene expression in formalin fixed paraffin embedded (FFPE) tissue samples. For this study a SnSAG2 probe was designed for *S. neurona* detection and IL-10 and IFNγ probes were also designed (Table II). All probe design was conducted by the ACD Biotech (Newark, California) bioinformatics team but the general structure of a probe resembles a Z and consist of 18-25 base region which complements the RNA sequence of interest, followed by a spacer region and then a tail sequence which
serves as a binding site for the pre-amplifier molecule used in the assay when two probes hybridize together. For the EPM group the FFPE samples sections were replicate of those used for IHC staining and close to where pathological lesion was identified. The manufacturers protocol for the multiplex fluorescent assay was followed. Briefly, samples were deparaffinized in xylene, dehydrated in 100% ethanol, endogenous peroxidases were inhibited with RNAscope kit specific reagent, antigen retrieval step consisted of submerging slides in boiling Antigen reterival reagent (ACD Biotech), followed by treatment with protease plus reagent provided in kit, slides were incubate at 40 C for 30 minutes with protease plus, washed with 1x RNAscope wash buffer. The probes were diluted appropriately in dilution buffer (included in kit). and incubated at 40 C for 2 hrs and then washed and incubated with pre amplifier 1, 2, and 3 in three separate 30 min (pre-amplifier 1 and 2) and 15 mins (pre-amplifier 3) incubations at 40 C, then amplifiers incubation for 15 mins followed by probe label ( TSA dye 520, 570 or 650) for 30 mins, this was repeated twice for amplifiers 2 and 3. In between each incubation period slides were washed 2x with RNAscope wash buffer. The final step was nuclear counter stain DAPI and cover slipping, air dry in the dark at room temperature for 30 mins and stored a 4 C. All slides were imaged with TiE- Nikon fluorescent light microscope utilizing the same light exposure sections for imaging consistency within 48 hours of staining.

**Table II: RNAscope probe sequence reference numbers**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence Ref number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SnSAG2</td>
<td>AY191006.1</td>
</tr>
<tr>
<td>IFNy</td>
<td>NC_009149.3</td>
</tr>
<tr>
<td>IL-10</td>
<td>NC_009148.3</td>
</tr>
</tbody>
</table>

**Statistical Analysis**
A two-ANOVA post hoc comparisons analyzed by Tukeys T- test was used for grouped analysis. Pearsons correlation test was used to determine correlation of cytokine and cell localization. All statistical analysis was done using GraphPad Prism Software V8.

Results

Immune phenotyping serum and CSF samples

Figure 1: A. The CSF concentrations and B. serum concentrations of IL-1β, TNFα, CCL2, CCL3, CCL11 and CCL5. C. CSF concentrations and D. serum concentrations of IFNα, IL-4, IFNγ, IL-17 and IL-10. There were no significant differences between the EPM group and control group of these measured parameters. D. CSF concentrations of soluble CD14 and E. Serum concentrations of soluble CD14. There was significantly more sCD14 in the CSF of EPM horses compared to control horses. All data is presented as ± SEM.
The cytokine/chemokine multiplex assay revealed no significant differences between the control group and EPM group in either to serum or CSF samples (Figure 1A,B). The CSF contained highest concentrations of CCL2 and CCL11 in both groups and the serum contained highest concentrations of CCL 3, 11 and 5. The multiplex cytokine assay also demonstrated no significant differences in the measured cytokines, between the two groups (Figure 1 C,D). The serum contained more cytokines than the CSF. There was a significantly greater amount of sCD14 in the CSF collected from EPM horses compared to control horses but there were no differences in serum concentrations (Figure 1E). The insignificant differences observed in the measured cytokines and chemokines may have been a result of sample size. The molecule sCD14 is associated with inflammation and given that the CNS pathology associated with EPM is thought to be of an inflammatory origin it makes sense that there would be an increase in sCD14 in the CSF.

**Histological Analysis (Pathology and IHC)**

| Figure 2. Representative IHC images from two EPM affected horses, dark brown staining encircled in black is *S. neurona* positive staining. |

Pathological lesions were identified in the 9 EPM affected horses in the current study with lesions varying in location and severity. The central nervous system was identified
to be pathologically normal in 9 control horses entered into this study. IHC results demonstrated *S. neurona* in 5 of 9 EPM affected horses. It is not always the case that *S. neurona* is found at lesion sites so it was not expected that all IHC results would be positive. Likewise, it may be that if additional IHC staining were completed *S. neurona* may be found.

**Immunofluorescence slide staining**

The quantitated cytokine values obtained from each captured 20x HPF image for all cell marker types and cytokine combinations analyzed (Table I) were plotted to determine correlation between cytokine and cell type localization in analyzed spinal cord samples (Figures 4 and 5). The only significant correlations observed were in the control group, there were significant positive correlation between the localization of astrocytes and IFNγ and IL-4 (figure 5 A & C).

For both the control group and EPM group the most

![Figure 3: For all immunofluorescent slide imaging all slides were imaged using a Nikon Ti-E fluorescent light microscope. All images were captured at 20x magnification and light exposure settings were kept consistent for all slides analyzed. For all images blue is nuclear staining (DAPI), A. Red is CD8 and pink is IFNγ. B. Green is CD4 and red is IFNγ. C. Green is glial fibrillary acidic protein (GFAP), red is connexin 43 (Cx43) which were both used to identify astrocytes and pink is IFNγ. D. Green is ionized calcium binding adaptor molecule-1 (Iba-1) which was used to identify microglia cells and pink is IL-17a.](image)
prevalent cell types were astrocytes followed by microglia which is not surprising as these are resident CNS immune cells and therefore found in greater abundance than T-lymphocytes under normal physiological conditions. It was hypothesized that more T lymphocytes would be observed in cytokine images collected from EPM affected horses, but this was not observed. While all antibody markers stained positively as depicted in figure 3A-D, we were not able to detect *S. neurona* in the frozen tissue samples and therefore we cannot say conclusively that our results reflect *S. neurona* induced pathology changes.
**Figure 4:** Correlation plot of localization of cell types and cytokines analyzed in EPM group. The Y axis depicts the score and the X axis the total number of cytokine in each image captured. There were no significant correlations observed.

**Figure 5:** Correlation plot of localization of cell types and cytokines analyzed in the control group. The Y axis depicts the score and the X axis the total number of cytokine in each image captured. There was significant positive correlation between astrocytes and A. IFNγ (P=0.0007) and C. IL-4 (P=0.04).
**RNAscope**
Utilizing samples collected for the current study we were able to validate the SnSAG2 probe to provide additional in-situ techniques for further studies. The SAG2 probe positively identified *S. neurona* in a cerebellum collected from an *S. neurona* infected mouse from another study. The cerebellum sample contained an abundant amount of *S. neurona* which was identified by IHC and then RNAscope. Likewise, *S. neurona* was found by RNAscope in four EPM horses which also demonstrated positive IHC staining. The IFNγ and IL-10 probes were used in combination with the SAG2 probe and in some cases IFNγ was visualized with the SAG2 probe but IL-10 was not observed with the SAG2 probe. Samples from the control group of horses which were identified by IHC to be negative for *S. neurona* were also negative by RNAscope for the SAG2 probe.

![Figure 6: RNAscope image of SnSAG2 staining (pink), IFNγ (green), cell nucleus (blue). A. positive control mouse cerebellum. B. Spinal cord tissue from EPM affected horse.](image)
These results help to validate the SAG2 probe adding to the techniques which can be utilized to examine the pathology of EPM affected horses.

**Discussion**

In the current study a variety of methods were utilized to explore methodology which could be used to investigate the immune response in the EPM affected horse. One of the main factors that limits EPM prognosis in affected horses is the lack of understanding of the immune response against the etiological agent *S. neurona*. This lack of knowledge impedes treatment and supportive therapy options. The current study investigated both the peripheral immune response and the immune response within the CNS, with a more detailed focus on the CNS, in a small population of EPM affected horses. The immunofluorescent analysis of IFNγ, IL-17a, IL-10 and IL-4 and cell markers for astrocytes, microglia and T lymphocytes were conducted in frozen tissue samples as opposed to FFPE tissue to negate the antibody interference/specificity issues that sometimes result from the cross linking of formalin fixed proteins (Delcambre et al, 2016). All selected antibodies for this study stained appropriately in the frozen tissue samples. However, because the frozen tissue sample sections were only from the same anatomical region (ex. C1) of histologically identified EPM lesions and not direct replicate sections, it is difficult to directly correlate these results with identified lesions. Since it was the goal of this study to evaluate the immunopathology in EPM affected horses this was considered a major pitfall. On the other hand, it is unknown how the immunopathology is altered in tissue surrounding pathological lesions and knowledge of this pathological aspect was gained in the current study. In the examined frozen tissue samples, we didn’t observe any significant differences in the cells which were present in
tissue samples collected from EPM affected horses compared to control horses. The cytokine’s analyzed were found in relatively the same abundance in both groups as well. The cytokine IFNγ was found most frequently in captured images of frozen tissue samples and IL-17a was found least frequently. There was a significant correlation between the localization of astrocytes and IFNγ and IL-4 in the control group but when interrupting these results, it should be considered that astrocytes were most frequently found in all tissue samples analyzed. In normal pathology astrocytes and microglia cells are found in higher abundance than T lymphocytes within the CNS because they are resident immune cells. Given that results of the current study did not significantly differ between control and EPM horses it is suggested that pathology in tissue surrounding pathological lesion site, may not be affected and therefore is not the optimal tissue sample for EPM immunopathology investigation. The ability to more closely correlate immunopathology analysis such as what was conducted in this study, with S. neurona itself or histological inflammatory lesions consistent with EPM would be ideal. To help expand on the tools available for immunopathology analysis we employed a novel in-situ hybridization technique, RNAscope. We successfully utilized a target probe for SnSAG2 to identify S. neurona in FFPE tissue samples collected from the EPM group which also positively stained for S. neurona by IHC analysis. The SnSAG2 sequence was selected for S. neurona probe design due to it being found on 7 different S. neurona isolates from horses (Howe et al, 2008) and its high specificity and sensitivity when used as a target protein for S. neurona antibody detection in ELISA (Yeagan et al, 2011). RNAscope allows visualization of gene expression instead of protein expression. The design of target probes is dependent on available gene sequence knowledge and is not limited by
available equine antibodies like traditional immunohistochemistry, which immensely expands the immune phenotyping possibilities in the horse. The use of FFPE tissues as opposed to frozen tissue samples allows for more extensive collaboration efforts of EPM researchers because FFPE tissue is routinely collected in postmortem examination whereas frozen tissue is not.

There were no significant differences observed in sera or CSF concentrations of measured cytokines and chemokines but there was significantly greater amount of sCD14 in CSF samples collected from EPM horses compared to control horses. The molecule sCD14 is increased during systemic inflammatory conditions which involve monocyte recruitment and macrophage activation in both the horse and human (Nockher et al, 1999 and Wagner, 2013). It has also been reported to be increased in inflammatory conditions which affect the CNS such as bacterial mengitis in humans (Nockher et al, 1999). Other biomarkers related to CNS damage such as phosphorylated neurofilament H (pNF-H) have been investigated as potential biomarkers for EPM (Intan-Shameha et al, 2017 and Gomez et al, 2019). The sCD14 results of the current study suggest that it too could potentially be used a biomarker for EPM diagnosis and future studies involving larger sample populations are warranted. Equine protozoal myeloencephalitis can acutely or chronically affect horses and treatment failure can result in disease relapse (Mackay et al, 2006). A biomarker such as sCD14 may also aid in monitoring disease status after treatment to help decrease potential for relapse. While there were no significant differences in analyzed chemokines and cytokines in the current study, this may have been a result of the small sample population and future studies utilizing larger study populations to examine changes in these cytokines and chemokines should be conducted.
If differences could be detected in these immune mediators, valuable information of immune cell recruitment and immune response (inflammatory or anti-inflammatory) could be gained. Overall the results of the current study suggest that the novel in-situ technique RNAscope may be optimal for studying the immunopathology of EPM. The use of this assay allows for *S. neurona* detection and the analysis of three other markers which could consist of cell markers, immune mediators or cell structural components, in FFPE tissue samples. The results of the current study and previous studies investigating EPM immunopathogenesis are limited by sample size, making it difficult to assess immunological changes due disease status (acute, chronic or relapsing) or treatment status, leaving many unanswered questions. An assay which allows for an extensive immunopathology analysis, in tissue samples routinely collected during postmortem examination in EPM suspected horses, eases difficulties associated with collaborative efforts, creating new opportunities for EPM research.

References


Chapter VIII: Conclusions Future Directions

Equine Protozoal Myeloencephalitis (EPM), is a neurologic disease in horses considered to be the most common neurologic disease of horses, yet the knowledge of the disease remains fairly limited. The work conducted here in mouse models, combined with the work in the previous literature, implicates an essential role of cytokine, interferon gamma (IFNγ), and IFNγ competent T lymphocytes, in the protective immune response against *S. neurona* induced encephalitis. The protective immune response results in control of *S. neurona* replication and elimination of the parasite before causing significant central nervous system (CNS) damage and subsequent clinical neurologic disease. In the immunocompromised mouse, this immune response is not limited to the role of T lymphocytes, as Balb/cscid mice which are void of T-lymphocytes, do not consistently succumb to neurologic disease after *S. neurona* infection. In has been reported that *in-vitro* bacterial stimulation of Balb/c splenocytes results in an increased production of innate immune cell derived IFNγ, compared to C57Bl/6 (B6) splenocytes (Koo, 2006). However, an increase in IFNγ production was not detected in the sera of Balb/c scid mice compared to C57Bl/6 (B6) *scid* mice 7 days post infection with *S. neurona*. If robust IFNγ production did occur in the Balb/c *scid* mice during this study, it may have occurred prior to the 7DPI sampling point or IFNγ may not have been the explanation for the resistance observed in these mice. Another immunological factor which differs between these two mice strains is their MHC haplotype. The MHC complex (I and II) are responsible for antigen presentation and are integral part of activating than robust immune response against an invading pathogen and adaptive immune response activation. The genetic variation between the B6 and Balb/c MHC haplotype is one contributing factor to *Toxoplasmosis* resistance in the Balb/c mouse compared to susceptible B6 mouse. There have also been reported differences in disease susceptibility
differences in B6 Ifnγ−/- and Balb/c Ifnγ−/- mice, with the Balb/c strain remaining neurologically normal for slightly longer than B6 strain, especially as the infection dose of *S. neurona* decreased (Dubey, 2013). Future mouse model studies to investigate the MHC haplotype role in *S. neurona* disease susceptibility could include *S. neurona* infection of NOD scid mice which have the same MHC haplotype as disease resistant ICR scid mice (Marsh et al, 1997 and Sellon et al, 2004). Therefore, it’s hypothesized that NOD scid mice would be resistant to *S. neurona* encephalitis, if disease resistance in an immune compromised state is dependent on MHC haplotype. Also, a B6 scid mouse with the Balb/c MHC haplotype (H2d) could be infected with *S. neurona* and disease resistance would be expected, if the hypothesized role of the MHC haplotype is correct. The protective immune response against *S.neurona* encephalitis is not limited to MHC haplotype as both Balb/c and B6 mice are resistant to *S. neurona* infection (Rosypal et al, 2002) but as mentioned it could potentially be influenced by MHC haplotype. Additionally, a role of MHC haplotype in EPM susceptibility is one explanation for not being able to readily induce EPM in experimental infection studies.

If there is a specific MHC haplotype that is associated with clinical EPM susceptibility in horses, disease susceptibility may also be further influenced by strain of *S. neurona* which is ingested. There is a considerable amount of genetic variation noted in *S. neurona* (Asmundsson et al, 2006). The amount of *S. neurona* ingested, either in a single exposure or multiple exposures, may also influence susceptibility. Another mouse model study which could be conducted is to test disease susceptibility in a mouse with just a CD8 knockout mutation to determine if the susceptibility reported in β2m knockout mice was strictly due to lack of functional CD8+ T lymphocytes or combined role of lack of β2m and MHC I expression and CD8+ T lymphocytes. If CD8 knockout mice are disease resistant, then susceptibility can be
attributed to β2m deficiency and immunologic impairment related to antigen presentation by MHC I complex. It is likely that CD8 knockout mice would be susceptible to disease as these cells are a main source of IFNγ production. Transfer of IFNγ producing CD3+ T lymphocytes offered protection against *S. neurona* infection in susceptible B6 *scid* mice. It would also be interesting to investigate disease outcome in a Balb/c β2m knockout mouse and determine if disease progression paralleled B6 β2m knockout mouse to further investigate the role of MHC complex in disease susceptibility. In addition to these studies further treatment studies should be conducted.

The B6 *Ifnγ* -/- mouse model was employed to investigate the treatment efficacy of antiprotozoal drugs diclazuril and decoquinate. Decoquinate was not proven to be effective at preventing infection despite different administration methods and confirmation that the mice received decoquinate during treatment periods. Decoquinate is a “sticky” compound with a short half-life and poor bioavailability, making it difficult to administer and potentially, pharmacologically unfavorable. A clinical field trial did report improvement in horses potentially affected by EPM after treatment with decoquinate, but the compound used for treatment was a combination of levamisole and decoquinate and individual drug testing was not completed (Ellison, 2012). Therefore, further studies investigating the efficacy of decoquinate and levamisole individually need to be conducted, as it could be the synergistic effect of these two compounds that resulted in neurologic improvement in Ellison et al (2012) study. Likewise, pharmacokinetic studies should be conducted to further test the bioavailability of the drug in the horse and determine if effective CNS concentrations can be obtained.

Further studies investigating immunomodulators which could serve as supportive therapy to help prevent relapses potentially associated with treatment failure should be conducted as
One immunomodulator that has been reported to increase IFNγ production is Zylexis (inactivated parapoxvirus) (Horohov, 2008). The common EPM treatment diclazuril did prevent *S. neurona* encephalitis while B6 *Ifnγ−/−* mice were being treated but cessation of treatment led to disease development. This suggest that the medication inhibited *S. neurona* replication but an IFNγ dependent immune response was necessary for complete parasite elimination. This also supports the idea that some horse’s relapse after response to EPM treatment as a result of treatment failure, expressing a need for treatment options which fully eliminate *S. neurona*. 

Lastly, studies investigating the immune response within the CNS and associated neuropathology are limited. This is partly due to difficulty to obtain funding for studies with a population large enough to investigate differences in disease states. Some EPM affected horses which develop neurologic signs and are treated and appropriately respond to treatment are able to return their job. However, some horses don’t respond to treatment and disease progresses to the point where they need to be retired from work or euthanized due to poor quality of life. Why these differences occur could be unrelated to neuropathology, but a pre-existing condition could also make some horses more susceptible to EPM infection or explain differences in EPM affected horses. Concurrent equine motor neuron disease and EPM (due to neospora hughesi infection) has been reported (Finno, 2010). Also, it has been reported that workload of horses is positively correlated with serum concentrations of phosphorylated neurofilament – H which accumulates due to neuron axonal degeneration (Morales Gomez, 2019). This suggest that working (riding) horses may experience axonal degeneration as result of neuron damage that might occur as a result of riding. It could as be that the neuroinflammation associated with EPM pathology could trigger another neurologic disorder such as equine motor neuron disease. Investigating these potential phenomena could help determine prognosis of EPM horses. Overall future studies
investigating the potential for genetic predisposition to EPM and improving treatment options would be valuable.

References


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