Interpretation of the Detection of Antibodies to Sarcocystis neurona
in the serum and CSF of young horses

by

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Detection of Antibodies to *Sarcocystis neurona* in the serum and CSF of young horses

Anne Grimsley Cook, DVM

(ABSTRACT)

Horses that are exposed to *Sarcocystis neurona*, a causative agent of equine protozoal myeloencephalitis, produce antibodies that are detectable in serum by western blot (WB). A positive test is indicative of exposure to the organism. Positive tests in young horses can be complicated by the presence of maternal antibodies. Passive transfer of maternal antibodies to *S. neurona* from seropositive mares to their foals was evaluated. Foals were sampled at birth (presuckle), at 24 hours of age (postsuckle), and at monthly intervals. All foals sampled before suckling were seronegative. Thirty-three foals from 33 seropositive mares became seropositive with colostrum ingestion at 24 hours of age, confirming that passive transfer of *S. neurona* maternal antibodies occurs. Thirty-one of the 33 foals became seronegative by 9 months of age, with a mean seronegative conversion time of 4.2 months. These results indicate that evaluation of exposure to *S. neurona* by WB analysis of serum may be misleading in young horses.

Cerebrospinal fluid (CSF) samples from 15 neonatal (2-8 day) foals were examined for the presence of antibodies to *S. neurona* by WB analysis. Twelve of 13 foals that were seropositive were also CSF positive, suggesting that maternal antibodies to *S. neurona* cross the blood-CSF barrier in neonatal foals resulting in a positive CSF
WB. Repeat taps were performed on 5 of the foals which showed that the immunoreactivity of the western blot decreases over time. Two of the 5 foals were CSF negative at 83 and 84 days of age, with 1 foal still positive at 90 days, and 2 foals positive at 62 days. These results indicate that maternal antibodies to *S. neurona* in the CSF can confound WB results in neonatal foals up to several months of age.
DEDICATION

To my husband and trusted friend, Wesley, for inspiring me to become a better companion, friend, and veterinarian.

To my parents for their constant support and encouragement through all of my personal, academic, and equine pursuits.
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<tbody>
<tr>
<td>AO</td>
<td>atlanto-occipital</td>
</tr>
<tr>
<td>AQ</td>
<td>albumin quotient</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CVM</td>
<td>cervical vertebral malformation</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>EHV-1</td>
<td>equine herpesvirus myeloencephalitis</td>
</tr>
<tr>
<td>EMND</td>
<td>equine motor neuron disease</td>
</tr>
<tr>
<td>EPM</td>
<td>equine protozoal myelocencephalitis</td>
</tr>
<tr>
<td>FPT</td>
<td>failure of passive transfer</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LS</td>
<td>lumbosacral</td>
</tr>
<tr>
<td>NSAID</td>
<td>nonsteroidal-antiinflammatory drugs</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear cell</td>
</tr>
<tr>
<td>RID</td>
<td>radial immunodiffusion</td>
</tr>
<tr>
<td>TNFa</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<tr>
<td>WB</td>
<td>western blot</td>
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Introduction

Equine protozoal myelencephalitis (EPM), caused by the protozoal organism *Sarcocystis neurona*, is the most commonly diagnosed equine neurological disease in the United States (US) (Dubey et al., 2001). This disease may profoundly impact the horse industry in the US due to the prolonged and expensive treatment without a guaranteed cure, the apparent increasing prevalence of the condition, and the loss of functional use of the affected horse. It is estimated that direct costs for diagnosis and treatment of EPM for horses in the US range from $54.4 to $110.8 million per year (Dubey et al., 2001). EPM is currently diagnosed by performing a western blot (WB) on cerebrospinal fluid (CSF).

Extensive research on EPM has occurred in the last decade; however, there is still much information yet to be elucidated about this disease in the horse. EPM is diagnosed by detecting antibodies to *S. neurona* in CSF with the WB. Western blot analysis can also be performed on serum. A seropositive result illustrates the presence of *S. neurona* specific antibodies in the serum tested (Granstrom, 1995) and indicates exposure of a horse to *S. neurona*. Recent studies have demonstrated that 45-55% of the horse population across the US have been exposed to *S. neurona* at some time in their lives, such that they mount a measurable humoral immune response (MacKay, 1997). A seropositive result by WB analysis is considered good evidence of exposure, but does not correlate well with the clinical diagnosis of EPM and cannot predict the risk of development of the disease.

The equine has a diffuse epithelialochorial placenta which prevents transplacental passage of large molecules. Therefore, a foal at birth is essentially agammaglobulinemic,
and maternal transfer of immunity in the horse occurs by ingestion and absorption of antibody-rich colostrum after birth. After ingestion of colostrum, immunoglobulin levels in the foal can be quantitated.

Currently, no studies have been performed on the specific transfer of EPM antibodies from seropositive mares to their foals. Presumably, a mare that is seropositive for EPM, and thus has circulating antibodies to *S. neurona*, would pass these antibodies to her foal in the colostrum. Many horses are “screened” for EPM by first performing a Western blot on serum. It is important to know when maternal antibodies to *S. neurona* are metabolized, thus when the foal becomes seronegative. Additionally, it has been established that neonatal foals have a more permeable blood-CSF barrier than adult horses, which results in higher concentrations of immunoglobulins in the foal’s CSF. Maternal antibodies to *S. neurona*, if ingested in colostrum, may pass into the CSF, resulting in a false positive WB test.

Two projects were undertaken to address these concerns about the *S. neurona* WB testing in young foals. The first project addressed whether passive transfer of maternal antibodies to *S. neurona* occurs, and if so, the length of time the maternal antibodies persist in the foal. This established the time frame for accurate serologic screening. The second project addressed whether these maternal antibodies to *S. neurona* cross the blood-CSF barrier after colostrum ingestion. If this occurred and the CSF was WB positive, this could profoundly affect the current methods of testing for EPM in young foals.
Chapter 1. Equine Protozoal Myeloencephalitis

1.1 History/Life Cycle

The syndrome of focal myelitis-encephalitis with characteristic histopathological lesions was first described in the 1960’s (Rooney et al., 1970). The protozoal origin of the disease was not identified until 1974 (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974), when the organism was recognized histologically as “Toxoplasma-like.” The etiologic agent was further characterized based on antigenic properties and named Sarcocystis neurona in 1991 (Dubey et al., 1991). In 1993, Granstrom et al. developed a WB specific to S. neurona for serum and CSF for antemortem diagnosis (Granstrom et al., 1993). The life cycle and transmission of S. neurona was undetermined until Fenger et al. (1995) proposed the opposum (Didelphis virginiana) as the definitive host. In 1997, in a cross-infection experiment, Fenger et al. (1997) induced clinical EPM by feeding sporocysts from opposums to horses; however, S. neurona was not demonstrated histologically or by cell culture. In 1998 (Dubey and Lindsay), the opposum was proven to be the definitive host for S. neurona by fulfilling Koch’s postulates in mice. After some controversy, S. neurona was conclusively differentiated from S. falcatula (Lindsay et al., 1999a; Cutler et al., 1999). The complete life cycle of the organism eluded many researchers until Dubey et al. (2000) determined that domestic cats, and perhaps armadillos, are an experimental intermediate host.

The proposed life cycle of S. neurona includes opossums as the definitive host, the domestic cat as the natural intermediate host, and a range of mammals as aberrant
Sarcocystis neurona infections have been diagnosed using immunohistochemical methods in cats, mink, skunks, raccoons, zebras, sea lions, and sea otters (Dubey et al., 2001; Dubey and Hamir, 2000). Clinical EPM in horses has been reported in the Americas, Brazil, Canada, and Panama (Masri et al., 1992; Clark et al., 1981; Granstrom et al., 1992).

*Sarcocystis neurona* belongs to the phylum Apicomplexa, which includes several genera that require a predator-prey life cycle. As with other *Sarcocystis* species, *S. neurona* has a heteroxenous life cycle, with sexual reproduction occurring in the opossum and asexual division with systemic infection in the intermediate host. The definitive host becomes infected by ingesting mature intramuscular cysts (with bradyzoites) from infected intermediate hosts. Bradyzoites penetrate the lamnia propria of the small intestine and form gametes (Dubey, 1976). By sexually reproducing in the intestinal wall of the opossum, *S. neurona* produce fragile, sporulated oocysts, which often rupture before being passed in the feces. Shedding of oocysts occurs for weeks to months (Fenger, 1997a). Sporocysts are not infectious to definitive hosts.
Figure 1. Life cycle of *Sarcocystis neurona* (Dubey et al., 2001) Copied with permission.
Exposure of the intermediate or aberrant host occurs through fecal contamination of the environment and of the food and water supply with infective sporocysts. After ingestion, the sporocysts penetrate vascular endothelial cells of the intestinal tract where they reproduce asexually (merogony) to produce merozoites which migrate to the central nervous system, where further asexual reproduction occurs intracellularly in neurons and microglial cells. To enter the central nervous system (CNS), the merozoites must penetrate the blood brain barrier (BBB). It is unknown whether they cross within leukocytes or directly through the cytoplasm of endothelial cells (Granstrom and Saville, 1998).

Histologic examination and cultures have played an important role in identifying parasite stages and site of occurrence. As with an intermediate host, only the asexual stages of *S. neurona* have been detected in the horse. However, sarcocysts from *S. neurona* have not been detected in equine muscle tissue, in contrast to their presence in muscle of typical intermediate hosts. The horse, therefore, appears to be a dead-end host (Fayer et al., 1990; Fenger, 1997a). Consequently, EPM is considered to be infectious, but not contagious, in that the disease cannot be spread directly from horse to horse. Transplacental infection has not been reported (Granstrom, 1993; Fenger, 1997b; Granstrom and Saville, 1998), but cannot be ruled out, especially since transplacental infections do occur with other closely related protozoa, such as *Toxoplasma*. While the most common cause of EPM is *S. neurona*, *Neospora* sp has been identified in 4 cases of EPM (Dubey et al., 2001).
1.2 Clinical Signs

The clinical signs of EPM are caused by both direct neuronal damage by the parasite and indirect damage by inflammation, usually resulting in progressive, asymmetric ataxia and focal muscle atrophy. *Sarcocystis neurona* can invade all regions of the CNS; therefore, clinical signs can vary widely, mimicking other neurologic conditions. The onset of EPM can be peracute, acute, or chronic, and the signs can range from focal to multifocal, or diffuse. Ataxia and incoordination can be present in one limb or all of the other limbs. Damage to lower motor neurons of the spinal cord or brainstem can result in muscle atrophy. Obscure lameness may be the only abnormality. Signs of cranial nerve dysfunction may be present in over 10% of EPM cases, including atrophy of the temporal masseter muscles, atrophy of the tongue, vestibular signs, facial nerve paralysis, laryngeal hemiplegia, and dysphagia (Fenger, 1997a; MacKay et al., 1992). Urinary incontinence occurs uncommonly (Scarratt et al., 1999). Infections of the cerebrum and cerebellum are less common, often associated with seizure activity or obscure lameness. Autonomic nervous system dysfunction has not been reported. In experimental infections, the clinical severity is directly related to the number of sporocysts ingested (Fenger et al., 1997). Inherent ability to resist infection is most likely related to immunocompetence and environmental stress.

1.3 Differential diagnoses

Equine protozoal myeloencephalitis can mimic most neurologic conditions, so these must be differentiated based on neurologic exam and lesion localization. Cervical vertebral malformation (CVM) is a disease that affects young horses, causes weakness
and ataxia of all four limbs, and must be differentiated from EPM (Moore et al., 1995). Clinical signs of CVM are often more symmetrical than with EPM, and the hindlimbs are usually more affected than the forelimbs. Equine herpesvirus myeloencephalitis (EHV-1) has a rapid onset of progressive symmetric hindlimb weakness often with bladder dysfunction. EHV-1 is frequently associated with outbreaks of upper respiratory infections and abortions. Equine motor neuron disease (EMND) initially presents with muscle atrophy and may appear similar to EPM in the early stages. Polyneuritis equi usually presents with hyperesthesia and progressive ascending paralysis; cranial nerve deficits may be present. Verminous migration results in variable signs depending on the site of migration and can involve diffuse or multifocal brain/spinal cord lesions (Granstrom and Saville, 1998). Other causes of neurologic dysfunction that should be considered include the following: trauma, CNS abscessation, equine degenerative myelopathy, rabies, viral encephalitis, neurotoxicity, CNS neoplasia, and guttural pouch mycosis. Musculoskeletal causes of abnormal gait and atrophy should be thoroughly investigated as well.

1.4 Diagnosis

Equine protozoal myeloencephalitis is the most commonly diagnosed infectious equine neurologic disease in the US (Boy et al., 1990; Hamir et al., 1992; Dubey et al., 2001). A complete neurologic examination is crucial to reach an accurate diagnosis. A
complete lameness examination is often necessary as well, to rule out a primary musculoskeletal problem. Blood chemistries are usually normal.

The antemortem diagnosis of EPM is currently based on the presence of clinical/neurological signs and a positive WB assay on a sample of CSF. Due to the immunoprivileged nature of the CNS, antibodies present in the CSF are produced locally in response to the presence of antigen in the CNS. In the case of EPM, the presence of the protozoan *S. neurona* in the CNS would provoke a specific immune response, which would result in a positive WB.

Cerebrospinal fluid collection is essential for antemortem diagnosis. The lumbosacral (LS) space is the preferred site for collection over the atlantooccipital (AO) space, as CSF flows caudally, and thus, an AO tap may not be as reflective of spinal cord disease or inflammation (Andrews, 1998). Additionally, an AO tap requires general anesthesia which can be hazardous with a neurologic horse. Since the disease is often focal, cytology is usually normal. When abnormal, CSF reveals increased total protein concentrations and a mononuclear pleocytosis (Fenger, 1997a). Cytologic examination is also important to reveal blood contamination of the CSF, as test results can be affected.

**Western blot**

The immunoblot test for EPM was developed in 1991. It utilizes cultured merozoites to detect antibodies (immunoglobulin G-IgG) directed against proteins unique to *S. neurona* (Granstrom et al., 1993; Liang et al., 1998). The commercial test (Equine
Biodiagnostics, Inc) utilizes 10-20%, 15 cm polyacrylamide gradient gels for protein separation. The specific proteins used for diagnostic testing have mobilities of 14.5, 13 and 7 kD; immunodominant bands at 30 and 16kD are non-specific proteins. A positive WB indicates the presence of antigen specific antibodies; however, it is not capable of quantitating the amount of antibody present.

The sensitivity and specificity of the WB test are reported to be very high, approaching 90% (Cohen and MacKay, 1997). False negative test results rarely occur; however, some horses fail to respond to the *S. neurona* specific proteins identified by the WB (Granstrom, 1993), causing a false negative result. If the horse is tested before development of an adequate antibody response, the results will also be falsely negative. Other causes of false negatives include technical laboratory error, prior EPM drug therapy, and possibly corticosteroid therapy (Cohen and MacKay, 1997).

A false positive result occurs when the WB is positive and the horse does not have EPM. The most common cause of false positive CSF results is likely to be blood contamination at the time of collection (Dubey et al., 2001; Miller et al., 1999); additionally, false positives can also be caused by a compromised BBB, laboratory error, and infection with a cross reacting organism. Increased permeability of the BBB can occur with inflammatory conditions of the CNS (equine herpes myelitis, meningitis, encephalitis, trauma, cervical vertebral malformation, CNS abscess), which allows for leakage of serum proteins, including serum antibodies, into the CNS. If the horse has been exposed to *S. neurona*, it may be seropositive, in that it developed antibodies against the organism without development of the disease. The WB cannot distinguish between
antibodies produced intrathecally and circulating serum antibodies to *S. neurona*.

Therefore, sample contamination with peripheral blood, as can occur during CSF collection, can also cause a false positive by the presence of serum antibodies. Cytologic examination can reveal the presence of red blood cells, which indicates sample contamination has occurred, and thus, the risk for a false positive result is significantly increased. A recent study indicated that as few as 8 RBC/mm$^3$ can suggest enough blood contamination for a false positive CSF WB (Miller et al., 1999).

CSF indices, in the form of albumin quotient (AQ) and immunoglobulin G (IgG) index, are tests that can be used to differentiate between serum and intrathecal antibodies by assessment of the permeability of the BBB, and thus, can potentially decrease the number of false positives (Andrews et al., 1995). The AQ is the ratio of CSF albumin to serum albumin (figure 2); an increased AQ may indicate damage to the blood CSF barrier or blood contamination of a CSF sample. The IgG index is calculated from levels of CSF IgG and serum IgG (figure 2), using the albumin levels as a reference (Andrews et al, 1995). An increased IgG index indicates intrathecal antibody production. This is a potential means for quantitating antibody production. The usefulness of the indices has been questioned as the increase in IgG can be due to other diseases that clinically resemble EPM (Cohen and MacKay, 1997). The albumin quotient’s use as a determinant of blood contamination has been disputed as well, as in some horses with strongly immunoreactive blood, contamination with as little

**Table:**

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<tr>
<td>Albumin Quotient (AQ)</td>
<td>$\frac{\text{CSF albumin}}{\text{serum albumin}} \times 100$</td>
</tr>
<tr>
<td>IgG Index</td>
<td>$\frac{\text{CSF IgG}}{\text{serum IgG}} \times \frac{\text{serum albumin}}{\text{CSF albumin}}$</td>
</tr>
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**Figure 2.** CSF index calculations
as $10^{-3}$ μl blood can result in a false positive WB (Miller et al., 1999). Thus, these indices should be interpreted with caution.

Recent case reports have suggested that *Neospora* organisms may be a rare causative agent of EPM. In one case, Marsh et al. (1996b) found that WB analysis on CSF was positive for *S. neurona* antibodies. Histological and immunologic examinations isolated *Neospora* as the causative agent, suggesting the *Neospora* infections may result in false positives.

**Serology**

Western blot analysis can also be performed on serum. A seropositive result indicates the presence of *S. neurona* specific antibodies in the serum tested (Granstrom, 1995). Serology is, therefore, useful to indicate exposure of a horse to *S. neurona*. Recent studies have demonstrated that 45-55% of the horse population across the United States have been exposed to *S. neurona* at some time in their lives, resulting in a measurable humoral immune response (MacKay, 1997; Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997a). It has not been determined how long a horse remains seropositive after exposure or if it ever reverts to a seronegative status.

A seropositive result by WB analysis is considered good evidence of exposure, but does not correlate well with the clinical diagnosis of EPM and cannot predict the risk of development of the disease. It is not possible to distinguish between antibody titers resulting from routine exposure and those resulting from active disease (Granstrom,
1995). It is unknown how many seropositive horses exhibit clinical signs of the infection, although the prevalence of clinical EPM in the United States equine population is estimated to be about 1% (Dubey et al., 2001). Thus, despite the seemingly high exposure rate, the prevalence of clinical disease is low. While serologic evaluation is not very practical in the diagnosis of EPM, it is a useful tool for examining the rate of exposure.

**Other methods of testing**

A polymerase chain reaction (PCR) DNA diagnostic test for *S. neurona* has been developed to detect antigen of the parasite in CSF (Marsh et al., 1996a). With CSF samples from histologically confirmed cases, PCR demonstrated 83% sensitivity and 100% specificity. The PCR is dependent on the integrity of the target DNA sequence; however, a strong inflammation response may cause degradation of the parasite, decreasing test sensitivity (Granstrom, 1995). Additionally, the intact organism rarely enters the CSF. Thus, the test may be more useful in early stages of the disease, when PCR can be used primarily as an adjunct confirmatory test for questionable or suspicious results of WB testing.

An immunofluorescence test was also developed to test equine serum for antibodies. This test cannot differentiate exposure to *S. neurona* from *S. fayeri*, a common parasite of equine skeletal muscle, and thus, lacks the necessary specificity to be useful (Granstrom, 1995; Granstrom et al., 1993). Recently, a direct agglutination test has
been described to detect antibodies to *S. neurona* (Lindsay and Dubey, 2001). This test is currently being validated for use in horses.

**1.5 Pathology**

Lesions of *S. neurona* are confined to the CNS, with the spinal cord most often affected (Fayer et al., 1990; Rooney et al., 1970). On gross examination, multifocal areas of hemorrhage and discoloration are often visible. The lesions are microscopic to several centimeters in diameter. Histologically, focal to diffuse areas of mild to severe nonsuppurative inflammation and necrosis with multifocal to coalescing areas of hemorrhage are present (Cusick et al., 1974; Dubey et al., 1974;). Neural tissue is often infiltrated by mononuclear cells, with occasional giant cells, neutrophils, and eosinophils. Perivascular infiltrates are also often present, including mononuclear cells with both lymphocytes and plasma cells. Both gray and white matter can be affected. Chronic changes include marked tissue destruction, with astrocytosis and gliosis with loss of neuronal structure (Mayhew et al., 1978).

Individual organisms can be found extracellularly or intracellularly, not only present in the cytoplasm of neurons, but also in the cytoplasm of leukocytes and rarely in vascular endothelium. Groups of organisms (meronts) are usually found in cells, typically in a rosette pattern (Fayer et al., 1990; Mayhew et al., 1978). There are no reports of *S. neurona* infection in extra-neural tissues of horses (Dubey et al., 2001).
1.6 Treatment

Little information is available regarding the appropriate clinical course and effect of treatment of horses with EPM. The primary aspect of therapy for EPM is the use of antiprotozoal agents. Many horses improve with treatment; however, most do not improve to neurologically normal or athletically functional. The rate of clinical improvement in treated horses appears to be about 60-70% (Saville et al., 2000a; Fenger et al., 1997c). Currently, there are several treatment options available.

The traditional treatment of EPM involves the synergistic combination of potentiated sulfonamides (trimethoprim and a sulfonamide) and 2,4-diaminopyrimidine (pyrimethamine). Pyrimethamine and trimethoprim are inhibitors of diaminopyrimidine dihydrofolate reductase (DHFR), a component of the protozoal enzyme DHFR-thymidilate synthase critical to folate metabolism in apicomplexan protozoa (Fenger, 1997c). The effects of DHFR inhibitors are potentiated by sulfonamides, analogs of paraaminobenzoic acid, which compete in the production of dihydrofolate. These drugs are effective against *S. neurona* in cell culture (Lindsay and Dubey, 1999b). Protozoa that are susceptible to pyrimethamine can become resistant in the absence of sulfonamides. Usual treatment involves once daily oral administration of sulfadiazine (20mg/kg) and pyrimethamine (1 mg/kg). Most treatment regimens cost $200-$400/ month (Dubey et al., 2001). Current therapies involve treating one month past resolution of clinical signs or up to six months.
Pyrimethamine causes inhibition of cell division in rapidly dividing cells (bone marrow precursors and enterocytes). Acute toxicity with large overdoses can result in profound intestinal and neurologic signs; however, chronic toxicity is more common, due to the usually lengthy treatment. DHFR inhibitors impair folic acid absorption, causing folic acid deficiency. Normocytic, normochromic anemia is a possible side effect. Profound folate deficiency can occur with pregnancy, resulting in teratogenic effects. Recently, congenital defects have been recognized in foals of broodmares being treated during pregnancy for EPM (Toribio et al., 1998).

Coccidiostats such as diclazuril or toltrazuril are currently gaining favor and FDA approval as firstline treatments. Both have reported rates of clinical improvement up to 75% (Granstrom, 1997). Diclazuril, a benzacetanitride coccidiostat for poultry, has anti-
S. neurona activity in cell cultures (Lindsay and Dubey, 2000a; Dubey et al., 2001) and appears to have some clinical efficacy (Cohen, 1998; Dirikolu et al., 1999; Granstrom et al, 1997). Toltrazuril, another triazine derivative, has good absorption into the CSF, a long elimination time (48-72 hours), and appears to have potential efficacy for the treatment of EPM (Furr and Kennedy, 2001). A metabolite of toltrazuril, ponazuril, appears to also have efficacy against S. neurona, both clinically and in vitro (Lindsay et al., 2000). These coccidiostats appear to be very safe for use in the horse.

Nitazoxanide is another available drug that appears to be beneficial for treatment of EPM. While it is effective against S. neurona in cell culture, it does not achieve adequate CSF concentrations and appears to have a low therapeutic index (McClure and Palma, 1999).
Adjunctive therapies may also be necessary to reduce inflammation of the affected nervous tissue. Nonsteroidal-antiinflammatory drugs (NSAIDs) or dimethylsulfoxide (DMSO) are often administered with the initiation of treatment. As merozoites are dying in response to treatment, they can cause an inflammatory reaction that can result in worsening of neurologic signs. The use of corticosteroids is contraindicated as immunosuppression may allow for parasite multiplication (Bowman et al., 1992; Dubey et al., 1974; Cutler et al., 2000). Folic acid supplementation is often recommended to prevent hematologic and fetal development side effects. Protozoa cannot utilize the supplemented form.

A recent controlled retrospective study identified factors associated with clinical improvement and survival (Saville et al., 2000a). In this study, horses with EPM were 10 times more likely to improve with treatment than those untreated. Horses used for breeding or pleasure were less likely to improve than race or show horses. The majority of horses with mild neurologic deficits improved with treatment, compared with fewer of the moderate or severe clinical signs groups. The likelihood of survival was inversely related to severity of clinical signs at presentation. EPM horses that improved were 50 time more likely to survive than those showing no improvement. The recognition of relapse after discontinuation of treatment has increased. Anecdotal reported rates of relapse are 10-30% (Reed and Saville, 1996).
1.7 Epidemiology

A recent large retrospective case control study at performed at The Ohio State University provides the first controlled insight into risk factors associated with an increased risk for developing EPM. There was a significant association of age, primary use, season of admission, location of the premises near a river/creek, security of feed from exposure to wildlife, wildlife commonly seen on premises, prior diagnosis of EPM on the premises, and health events prior to admission (Saville et al., 2000b).

While the seroprevalence to *S. neurona* appears to increase with age, EPM appears to be a greater risk to young horses (Saville et al., 1997a). Age appeared to be an important risk factor in this Ohio study with the highest risk in young horses 1-5 years old. This finding is consistent with previous studies (Boy et al., 1990; Fayer et al., 1990). In a retrospective study, Boy reported 87.5% of clinical cases of EPM occurred in horses between 1 and 6 years of age (Boy et al., 1990). In a study of histologically confirmed cases, 61.8% were less than 4 years old, with the youngest reported horse in this study being 2 months old (Fayer et al., 1990). As with other infectious diseases, the risk appears to be higher among the younger animals that have not acquired protective immunity through exposure (Saville et al., 1997b).

Previous reports have suggested increased prevalence in Standardbreds and Thoroughbreds (Boy et al., 1990). It has been acknowledged that these breeds are observed more intensely at a younger age, thus abnormalities may be recognized earlier. Saville’s recent study is supportive of earlier findings, identifying Thoroughbreds,
Standardbreds, and Warmbloods as breeds at risk (Saville et al., 1997b). Fewer ponies have been reported with EPM, suggesting that ponies may be more resistant to the disease (Dubey and Miller, 1986; Fenger, 1997a).

The highest risk of disease in the Ohio study was seen in the warmer months (Saville et al., 2000b). This could be due to a climatic effects on exposure to *S. neurona* or increased stress in summer due to heat or competitions (Saville et al., 2000b). In a previous study, there was a correlation between geographic seroprevalence and number of freezing days (Saville et al., 1997a; Saville et al., 1997b), indicating that geographic variation is probably due to climactic variation.

It has been suggested that the geographic distribution of the seroprevalence of EPM closely matches that of the opossum (Fenger et al., 1995; Dubey et al., 2001). EPM has been reported in North, Central, and South America and appears to be limited to these areas. Case reports from Europe and Africa involved horses exported from the Americas (Mayhew and Greiner, 1986; Ronen, 1992; Lam et al., 1999).

The Ohio study found that use of horses in races and shows was associated with increased risk of developing EPM (Saville et al., 2000b). High intensity exercise (Hines et al., 1996) as well as the stress of competition can suppress the immune system in the horse. Similarly, health events, such as injury, surgery, or parturition can result in immunosuppression, as supported by the data from the Ohio study.

Onset of clinical signs is often associated with these periods of increased stress, such as intense training, pregnancy, and transport, resulting in an incubation period that is
quite variable. Fayer’s histologic confirmation of EPM in a 2 month old foal suggests that the minimum incubation period may be 2 months (Fayer et al., 1990). Experimental induction of EPM using large numbers of sporocysts resulted in clinical disease by 28-42 days (Fenger et al., 1997). Clinical disease has exceeded 1 to 2 years after exportation of some horses, suggesting that the protozoan can remain in the body for extended periods of time without causing noticeable clinical disease (Mayhew and Greiner, 1986).

1.8 Prevention

Of the risk factors associated with EPM (Saville et al., 2000b), several identified were associated with management (see epidemiology). Manipulation of some of these risk factors may help to reduce the risk of EPM on a farm. In association with the life cycle of *S. neurona*, the most direct prevention of EPM is to limit the access of opossums to the food and water sources of horses. Grain, fruit trees, spoiled meat, and garbage are common attractants for opossums, and elimination of these food sources is paramount to their control. Dead animals should be disposed of quickly and properly. Food should be secured in an area with limited access.

Development of an efficacious vaccine against *S. neurona* is a highly desired step toward prevention of EPM. However, there has been little success in developing a potent vaccine against other protozoan parasites. A killed vaccine is currently in the initial stages of distribution. While its safety has been established, its efficacy is unknown.
Chapter 2: Passive Transfer of Maternal Antibodies to *Sarcocystis neurona*

2.1 Passive Transfer and Immunologic Considerations in the Neonatal Foal

2.1.1 General

Immunoglobulin derived from colostrum is one of the most important factors in determining the morbidity and mortality of foals (Naylor, 1979). The neonatal foal before suckling is fundamentally agammaglobulinemic (Jeffcott, 1975; LeBlanc, 1990) and is susceptible to infective microorganisms in the environment. The high incidence of infections in foals with low serum immunoglobulin G (IgG) concentrations substantiates the importance of colostral transfer of antibody in neonatal foals.

In 1924, it was demonstrated that no significant placental transmission of antibodies in the horse occurs during gestation (Jeffcott, 1975). The equine placenta is characterized as diffuse epitheliochorial with six cell layers separating maternal and fetal circulations, including the maternal and fetal endothelia, maternal and fetal connective tissue, uterine epithelium, trophoblast cells, and four basement membranes (Flood, 1993). The persistence of the three uterine layers of the mare prevents transplacental passage of large molecules.
2.1.2 Colostrum

The mammary gland selectively concentrates immunoglobulins from the blood just prior to parturition as a result of changing progesterone and estrogen levels (Jeffcott, 1975). A Fc receptor on acinar epithelial cells in the mammary gland transports IgG into the colostrum (Butler, 1998). Levels in colostrum can be 2 to 4 times higher than that of serum. This is evidenced by a significant fall in the mare’s serum globulin levels about two weeks prior to foaling (Jeffcott, 1974b). The mechanism of this selective concentration is unknown, and the concentration of immunoglobulin in the colostrum of the mare is variable. Colostrum secretion is short-lived, lasting less than 24 hours postpartum for most mares (Jeffcott, 1974b). Mares produce an average of 1.5- 2 liters of colostrum (LeBlanc, 1990). The rate of colostrum secretion has not been well documented and appears to vary greatly amongst mares, due to breed, age, nutritional status, milk let-down, and ease of milking (Lavoie et al, 1989). One study of colostrum secretion in pony mares reported a mean rate of 160 ml/ hour in the first 24 hours post foaling (Jeffcott, 1975), while a study of horse mares reported a rate of 202-389 ml/ hr (Lavoie et al, 1989). Regardless, the level of immunoglobulin secretion falls rapidly with suckling. Samples collected 12-15 hours after birth have approximately 15% of the concentration at parturition (Jeffcott, 1974b).

The predominant immunoglobulins present in colostrum are IgG (1500-5000 mg/dl) and IgGT (500-2500 mg/dl), with lower levels of IgM (100-350 mg/dl), IgA (500-1500 mg/dl), and aggregating immunoglobulin (Al) (Jeffcott, 1975; MacDougall, 1975; Kruse Elliot and Wagner, 1984). IgGb is the most abundant subclass of IgG in colostrum.
IgA concentration rises during lactation and is the predominant immunoglobulin in milk at 16 days postpartum (Naylor, 1979). The other immunoglobulin levels rapidly decline when colostrum secretion ceases (Jeffcott, 1975).

The foal must ingest colostrum within the first 24 hours of life, preferably during the first 6 hours, in order for immunoglobulin absorption to occur. Absorption is maximal after birth but is progressively reduced over 24 hours. At birth, specialized epithelial cells of the small intestine nonselectively absorb the colostral proteins by pinocytosis. A specialized Fc receptor on the mucosa epithelial cells, present in most mammals and presumably in the foal, binds the colostral immunoglobulins and initiates the active transport process (Tizard, 1996). Within 24-38 hours, these cells are replaced by more mature cells of the intestine which are unable to absorb macromolecules (Jeffcott, 1975). Lower molecular weight proteins are also absorbed with the immunoglobulins; these are primarily the “milk proteins” β-lactoglobulin and α-lactalbumin which are produced by the mammary gland and are of no immunologic value. Because of their small size, they are rapidly excreted by the kidney, producing a transient proteinuria (Jeffcott, 1975). The large immunoglobulins travel via lymphatics to reach systemic circulation (Jeffcott, 1975). Peak absorption is reached about 6 hours after ingestion, so that by 18 hours of life, peak values of passive immunoglobulin levels are attained (Jeffcott, 1974b). It has been shown that the absorptive efficiency of the gut at a point in time is inversely related to the amount of colostrum ingested (Jeffcott, 1972). It has also been suggested that factors present in the milk can induce closure of the gut. Increased turnover rate of
intestinal cells after birth may be systemically triggered by the high levels of circulating adrenal corticoids (Jeffcott, 1975).

The minimum concentration of IgG considered to be adequate ranges from 400-800 mg/dl. The lower range may be sufficient to protect a foal in a clean environment with minimal exposure to pathogens (McGuire et al, 1977); IgG concentration >800 mg/dl is considered optimal. In a study of foals presented with septicemia, the average IgG level was 400 mg/dL; none of the infected foals had IgG levels >800 mg/dL. Thus, the study concluded that foals with an IgG plasma level of 400-800 mg/dL were at a much higher risk of acquiring infection than foals with >800mg/dl (Brewer & Koterba, 1988). This study was important as it raised the level of “adequate” passive transfer to a higher standard.

Absorption of colostrum may be accelerated by the presence of “enhancement factors” in colostrum which enhance the efficiency of absorption of macromolecules (Jeffcott, 1975). These factors have not been isolated or characterized in the foal; in the calf, two groups of substances appear to be involved, a low molecular weight protein fraction, and inorganic phosphate and glucose-6-phosphate (Jeffcott, 1975). Low proteolytic activity in the GI tract may also aid in the absorption of colostral proteins (Kruse-Eliot & Wagner, 1984). While present in the colostrum of other large animal species, acid resistant trypsin inhibitors are not present in equine colostrum, and therefore, are not essential for transmission of maternal immunity as previously thought (Bainter & Csapo, 1996).
Colostral quality can be roughly estimated by its appearance. A “high quality” sample is usually thick, yellow, and adhesive. Immunoglobulin content of the colostrum can be quantitated, either indirectly by colostrometer measurement, or directly, by radial immunodiffusion. The colostrometer measures the specific gravity of the colostrum which is significantly correlated with the colostral IgG concentration (LeBlanc et al, 1986; Dascanio et al, 1997). In addition, foal serum IgG concentrations highly correlate with specific gravities of the colostrum ingested (LeBlanc et al, 1986). Ingested colostrum with a specific gravity >1.060, which correlates to an IgG concentration of >3000 mg/dL, will predictably result in foal serum IgG concentrations >520 mg/dL (LeBlanc et al, 1986). Detection of low colostral immunoglobulin concentrations immediately post parturition allows for identification of foals at high risk for failure of passive transfer.

The average colostral IgG concentration varies with breed. The highest mean levels have been documented in Arabians (6100 mg/dL) and the lowest in Standardbreds (4000mg/dL) (LeBlanc & Tran, 1987). In addition it has been shown that IgG (T) levels are much higher in the colostrum and serum of pony mares than heavier mares (Jeffcott, 1975). Despite these differences, there has not been a reported correlation with decreased risk of passive transfer in Arabians and ponies.

Colostrum can be banked and frozen for supplementation to high-risk foals. It will remain stable for 18 months at -20°C. Banked colostrum is ideally of high quality with specific gravity >1.090 (IgG>7000mg/dL) (LeBlanc et al, 1986) and from a well vaccinated mare in the same environment as the foal, thus providing protection against
potential local pathogens. Additionally, banked colostrum should be evaluated for alloantibodies that could result in neonatal isoerythrolysis.

Quantitative analysis of circulating immunoglobulins in the foal can be determined by radial immunodiffusion (RID), concentration immunoassay technology (CITE test), the Latex agglutination test, glutaraldehyde gel (Gamma Check tests), and the zinc sulfate turbidity test. RID allows for the most accurate and specific quantitation of IgG levels; however, it requires a 24 hour incubation period so it is not very practical when attempting to prevent FPT. The zinc sulfate turbidity test is a rapid, inexpensive test that can be performed in the field. The amount of precipitation (turbidity) is proportional to IgG concentration in the sample. It can be estimated visually or quantitatively with a spectrophotometer. While a good screening test, it is associated with a high incidence of false positives. The latex agglutination test is more accurate than the turbidity test but does not distinguish a foal with barely adequate passive transfer (400 mg/dl) from a foal with optimal IgG content (>800 mg/dl). The glutaraldehyde test is based on cross-linking of gamma globulins; the time required for gel formation is inversely proportional to the globulin concentration (Jones & Brook, 1994). While a good screening test in the field, it has poor sensitivity and specificity. The CITE test is an easy, rapid semiquantitative test that can be performed in the field and produces reliable results. The CITE test is currently the most frequently used screening test (LeBlanc, 1990). A SNAP Foal IgG test has recently been developed and is similar to the CITE test. It appears to be an accurate screening test for identifying foals with IgG<800mg/dl, although it tends to
underestimate some foals which may result in unnecessary therapy (Bertone et al., in press).²

2.1.3 Other Colostral Components

As stated, colostrum appears to contain components other than immunoglobulins which can be absorbed by the foal. Fat soluble vitamins, such as vitamins A and E, are present in colostrum 3-8 times higher than in milk. Levels of B-carotene are 65 times higher in colostrum (Schweigert & Gottwald, 1999). These substances can play a critical role in modifying the immune system. Selenium is able to cross the placenta, but is also present in high quantities in colostrum. A deficiency in vitamin A, E or selenium can result in increased susceptibility to infectious diseases (Lewis, 1995). Concentrations of mineral elements (Mg, Na, K, S, Cu, Fe, Zn) are higher in colostrum, while concentrations of Ca and P are higher in milk (Grace et al, 1999). Similarly, the amino acids cystine, glycine, serine, threonine are higher in colostrum, while glutamate, proline, methionine, isoleucine, and lysine are higher in milk (Davis et al., 1994).

Hormones, such as insulin-like growth factor I (IGF-I) and insulin are present in high concentrations in colostrum; the transfer of these hormones may be important in gastrointestinal tract development (Hess-Dudan et al, 1994; Slebodzinski et al., 1998). Epidermal growth factor (EGF)-like activity was found to be present in mares’ milk, with the greatest concentration in colostrum (Murray et al, 1992). EGF has potential gastric mucosal protective and healing functions in suckling neonatal foals. Triiodothyronine
(T3) levels are increased in colostrum and milk and may play a significant role in the process of intraluminal digestion, absorption and maturation of enzyme systems (Slebodzinski et al., 1998).

In a study involving endotoxin administration to neonatal foals, colostrum deprived foals had lower peak concentrations of interleukin (IL) 6 and tumor necrosis factor alpha (TNFa) than colostrum fed foals, as well as longer time to peak serum concentrations (Robinson et al, 1993b). The higher and more rapidly attained IL6 concentrations in the colostrum fed foals suggests colostrum ingestion has immunostimulatory effects that can serve to increase resistance to neonatal infections, especially those of gram negative bacteria.

Studies of human colostrum have shown the presence of cytokines (IL1, IL6, TNFa, interferon-gamma (IF-g)) (Bocci et al, 1991). Bovine colostrum appears to contain TNF and at least detectable amounts of IL 2 (Sordillo et al, 1991). These presumably are present to increase the activity and efficiency of the neonatal immune system. Concentrations of fibronectin were recently measured in healthy mares and their neonatal foals. There were no differences in fibronectin amounts among the mares’ plasma, the foals’ presuckle plasma samples, and the foals’ postsuckle plasma samples (Martens et al, 1991).
2.1.4 Failure of Passive Transfer

Failure of passive transfer (FPT) is one of the most common underlying factors in neonatal sepsis and bacterial infections in foals less than four weeks of age (McClure, 1993; Morris et al, 1985; Jeffcott, 1974), with an estimated incidence of as high as 10% (Raidal, 1996) -25% (Morris et al, 1985; Crawford & Perryman, 1980). Foals over 24 hours of age with serum concentrations of IgG <200 mg/dL are classified as having FPT. Serum values of 200-400 mg/dL are considered to have partial FPT. Without the protective immunity provided by maternal antibodies, the foal is susceptible to environmental pathogens and is significantly at risk for neonatal disease. Failure of passive transfer has been attributed to the following:

1) Premature onset of lactation: This results in poor colostral quality and is the most common factor in poor passive transfer (Pearson et al, 1984). Common causes include premature placental separation, twinning, and placentitis.

2) Failure of the mare to concentrate sufficient quantities of IgG in colostrum: Differences in colostral concentrations are likely due to age of the mare, the number of lactations, the yield of colostrum, herd management, and possibly breed (Pearson et al, 1984).

3) Failure of the foal to ingest a sufficient volume of colostrum within the first few hours of life: Prolonged time to suckling can be due to difficulty standing from orthopedic, neurologic, or metabolic diseases, as well as maternal rejection.
4) Failure of the foal to absorb IgG across the gastrointestinal tract: Insufficient IgG absorption is a poorly understood phenomenon. Low serum IgG levels have been observed in sick foals regardless of the quantity or quality of colostrum administered. It is unknown what the effects of stress and release of glucocorticoids may have on premature closure of the gut (Naylor, 1979; LeBlanc, 1990). One study in which ACTH was administered to newborn foals showed that elevated serum cortisol did not reduce the ability of the foal to absorb colostral IgG (Carrick et al, 1987). Other possible explanations include metabolic breakdown of immunoglobulins because of inadequate nutrition and sequestration of immunoglobulins in the extravascular space following absorption (LeBlanc, 1990).

If FPT is anticipated and the foal is <18-24 hours old, the foal can be supplemented with banked colostrum. At least 15 ml/kg of colostrum containing > 7000 mg/dl IgG or SG>1.090 should be administered. Reconstituted lyophilized hyperimmune equine serum can also be administered orally; 40-60g IgG is needed to attain serum IgG>800mg/dl (Shideler et al, 1987). Bovine colostrum has been used, although its half life is much shorter (7.4 days) and its level of protection is questionable (Holmes & Lunn, 1991). Finally, plasma can be fed orally if the intravenous route is not an option. If plasma is administered orally, 6-9 L may be necessary due to the lower IgG concentration and decreased absorption compared to colostrum (Lewis, 1995).

If FPT is recognized after 18 hours of age, a plasma transfusion is indicated. Plasma has been shown to augment the immune response in septic neonates. The quantity administered should be based on whether the treatment is prophylactic or whether
systemic infection is established. The serum half-life of IgG in the face of septicemia can be half of the value when treating prophylactically. The increased loss of IgG is presumably due to increased catabolism secondary to infection and increased use of IgG (White, 1988). Even when used prophylactically, 30% of exogenous IgG is lost by 7 days. Marked reduction is potentially due to clearance of denatured IgG, clearance of IgG complexes, change in the catabolic rate of IgG, or distribution to extravascular sites (White, 1988). Therefore, the amount of IgG needed in foals with FPT is dependent on the health in the foal, the risk factors associated with the particular foal, and the baseline level of IgG (White, 1988).

In a recent study of 334 foals to determine the incidence and risk factors of foal mortality, the most important risk factor for foal mortality was FPT, predominantly due to inadequate consumption (Haas et al, 1996). In a prospective study in which eight foals were completely colostrum-deprived in a well managed environment, seven foals became septicemic, while none of the control foals demonstrated any clinical signs of sepsis (Robinson et al, 1993a). This study illustrated the importance of colostral immunity in protecting foals from opportunistic and pathogenic bacteria.

It is also recognized that the prevalence of infectious diseases, their severity, and foal mortality are substantially affected by management and environmental practices. In a study on an extremely well managed farm, there was no difference during the first 21 or 90 days in disease incidence or severity, health, or survival between foals with IgG levels above or below either 400 or 800 mg/dl (Baldwin et al, 1991). This study demonstrates that management may be as important as insuring adequate IgG concentrations. Studies
in Kentucky demonstrated that a high survival rate can be achieved in hypogammaglobulinemic foals and that IgG concentrations are not related to the prevalence or severity of disease (Baldwin et al, 1991). Additionally, in a retrospective study identifying factors associated with survival in septic foals, the percentage of foals that survived and had adequate (>800 mg/dl) concentrations of circulating IgG (40%) was not significantly greater than that of foals with IgG concentrations <400 mg/dl (36.8%) or IgG concentration 400-799 mg/dl (54.6%) (Gayle et al, 1998).

2.1.5 Metabolism of Maternal Antibodies

Passive antibody levels decline rapidly in the first four weeks of life and are usually minimal by six months of age. The primary immunoglobulins present in colostrum are IgG and IgG (T), the respective half-lives of which are approximately 23 and 20 days (MacDougall, 1975). A recent study calculated serum half-lives of 32 days for IgGb and 21 days for IgG(T) (Sheoran et al., 2000). Maternal IgM disappears from the serum of the foal more rapidly, usually by 16 days of age (Naylor, 1979; McGuire & Crawford, 1973).

Multiple studies have been performed to determine the kinetics of maternal antibody metabolism specific to certain diseases. The rate of decline appears to vary for both individual foals and different infectious agents. As seen in figure 3, the concentration of maternal antibodies in foals is nonprotective at 2-3 months of age; however, the remaining antibody can still cause vaccine inference. It has been suggested
that frequent use of vaccines with persistent maternal antibodies can induce a state of tolerance, preventing protective responses to diseases such as eastern equine encephalitis (Wilson et al., 1995), equine influenza (van Maanen et al., 1992), equine herpes virus 1/4 (van Maanen et al., 1994). Using methods that result in quantitation of an antibody titer, the decay of maternal antibodies can be observed over time. For example, the half life of passively transferred antibodies to equine arteritis virus (EAV) was determined in a recent study to be 32 days; these antibodies were detectable up to 7 months by virus isolation (Hullinger et al., 1998). Table 1 contains information regarding reported times of disappearance of maternal antibody to specific disease in the horse.

![Serum Antibody in the Neonatal Foal](image)

**Figure 3. Metabolism of maternal antibodies and production of autogenous antibody in the foal (Crawford and Perryman, 1980)**
<table>
<thead>
<tr>
<th>Specific Antibody</th>
<th>Duration of Antibody</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine infectious anemia</td>
<td>2 months</td>
<td>McGuire &amp; Crawford, 1973</td>
</tr>
<tr>
<td></td>
<td>2 to 6 months</td>
<td>Burns, 1974</td>
</tr>
<tr>
<td>Eastern equine encephalitis</td>
<td>2-3 months</td>
<td>Gibbs et al., 1988</td>
</tr>
<tr>
<td></td>
<td>20 days</td>
<td>Ferguson et al., 1979</td>
</tr>
<tr>
<td>Equine influenza</td>
<td>2-4 weeks</td>
<td>Liu et al., 1985</td>
</tr>
<tr>
<td></td>
<td>3-6 months</td>
<td>Van Maanen et al., 1992</td>
</tr>
<tr>
<td>Equine herpesvirus</td>
<td>1 month</td>
<td>McGuire &amp; Crawford, 1973</td>
</tr>
<tr>
<td>Clostridium tetani</td>
<td>2.5 months</td>
<td>Liu et al., 1982</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
<td>Rossdale &amp; Scarnell, 1961</td>
</tr>
<tr>
<td></td>
<td>17 months</td>
<td>McGuire &amp; Crawford, 1973</td>
</tr>
<tr>
<td>Equine arteritis virus</td>
<td>7 months</td>
<td>Hullinger et al., 1998</td>
</tr>
<tr>
<td><em>Sarcocystis neurona</em></td>
<td>9 months</td>
<td>Cook et al., 2001</td>
</tr>
<tr>
<td>African horse sickness</td>
<td>7.5 months</td>
<td>Alexander &amp; Mason, 1941</td>
</tr>
</tbody>
</table>

Table 1. Reported durations of maternal antibodies to specific equine diseases.
2.1.6 Foal Immunity

Protection against disease during the first 4-8 weeks of life is provided by the passively acquired maternal antibodies and nonspecific host defense mechanisms (LeBlanc, 1990). Serum concentrations of immunoglobulins are lowest between one and two months of age, due to catabolism of maternal antibodies (figure 3). Endogenous levels slowly rise, but adult levels are usually not approached until about four to eight months of age (Jeffcott, 1975). Foals can be more susceptible to infections at this critical low point of serum IgG concentration. Colostrum deprived foals appear to show an earlier and more rapid rise in autogenous IgG. This may be the result of a functional immunosuppression associated with the natural ingestion of colostrum (Holmes & Lunn, 1991).

The lymphoid thymus is first seen in the equine fetus at 11-12 weeks of gestation. The mesenteric lymph nodes are observed macroscopically at 14 weeks of gestation. Lymphoid infiltration into the spleen, the last type of lymphoid tissue to begin development, is not noted until 25 weeks of gestation, (Mackenzie, 1975). It has been shown that T-lymphocytes are present at 100 days of gestation (Perryman et al, 1980). By 180 days of gestation, B-lymphocytes are present in the fetus and can produce and secrete immunoglobulins, primarily IgM and IgG (McGuire & Crawford, 1973; LeBlanc, 1990; Perryman et al, 1980). Small quantities of IgM (8-20 mg/dl) and occasionally trace amounts of IgG (<15mg/dL) are detectable in the serum of newborn foals before suckling and are considered endogenous in origin (Naylor, 1979; Perryman & McGuire, 1980; Kruse-Elliot & Wagner, 1984). The presence of significant quantities of IgG in serum of
newborn foals before suckling is highly suggestive of in utero exposure to antigen after 180 days of gestation.

The foal is immunocompetent at birth; however, appreciable levels of autogenous immunoglobulins are not usually attained until 2-3 months of age. If antigenic exposure and stimulation occurs immediately after birth, a primary immune response in the form of autogenous IgG will be detectable at about two weeks of age (McGuire & Crawford, 1973). The number of circulating B cells is about 1/3 of adult numbers until the foal is about 3 weeks of age (LeBlanc, 1990). The protective effects of maternal antibodies in foals are strongly illustrated by foals with combined immunodeficiency (CID). These foals appear clinically normal until about 1-2 months of age, when maternal antibodies are at their lowest levels. Without a functional lymphoid system and the protection of maternal antibodies, these CID foals rapidly die of fatal infections (McGuire et al, 1977).

Cell mediated immunity may be immature at birth, although it has not been completely assessed (LeBlanc, 1990). A recent study characterizing maturation of cellular immune responses illustrated that general immune function, mitogenic responses, LAK cell activity, opsonized phagocytosis, and oxidative burst activity of the neonate were similar to the adult, but lacked only in total immune cell numbers (Flaminio et al, 2000). Opsonic activity of foal serum with >600 mg/dL IgG appears to be similar to opsonic activity in adult serum, while opsonic activity of foal serum with < 350 mg/dL IgG is significantly lower than in adult serum (LeBlanc, 1990). Therefore, the foal with FPT does not have adequate concentrations of circulating opsonins; if it becomes exposed, it lacks the ability to effectively opsonize invading bacteria.
It has also been shown that equine polymorphonuclear cells (PMNs) are functionally mature at birth; however, PMNs obtained from newborn foals before ingestion of colostrum demonstrate deficient chemotactic and phagocytic activities when tested with autologous plasma (Bernoco et al, 1987). This study suggests that maternal transfer of immunoglobulins and other humoral components (complement) may play a role in activating chemotactic and phagocytic function of PMNs derived from the newborn foal (Bernoco et al, 1987). It has also been demonstrated that phagocytic function of neutrophils varies during the first month of life. At 2 weeks of age, a high functional rate is observed that gradually declines by 4 weeks of age (LeBlanc, 1990). This variation is poorly understood, but clinically it may predispose a foal to disease at 4-6 weeks of age, especially with waning maternal antibody.

Among mammals, complement activity develops in fetal life. Presuckle values of foals range from 13% to 50% of adult complement activity; adult values are obtained from 1 month to 5 months of age (Bernoco et al, 1994). In colostrum deprived foals, complement activity and C3 concentrations are significantly higher at 2-5 days of age than colostrum fed foals (Bernoco et al., 1994). This suggests not only that the high complement activity may provide an alternative in immune defenses in colostrum deprived foals, but also that maturation of complement activity in neonatal foals is colostrum independent (Bernoco et al., 1994).

Immunostimulants administered to pregnant mares appear to have effects on the fetus, resulting in immunostimulating effects on nonspecific immunological mechanisms. Foals whose dams were administered levamisole revealed a significant increase in
cellular defense mechanisms, manifested by an increase in PMN phagocytic activity capable of reducing NBT, an increased phagocytic index, and increased destructive ability of neutrophils (Krakowski et al., 1999). This high degree of phagocytic activity occurred before colostrum ingestion and lasted until about 5 weeks of age. Additionally, increases in lysozyme and total IgG levels, predominantly IgG(T), were noted in foals of levamisole treated mares. Similar results were found with 1,3/1,6 glucan (Krakowski et al., 1999).

2.1.7 Summary

The importance of colostral ingestion by the foal has been recognized for many years. Failure of passive transfer of maternal immunity has been established as a significant risk factor in equine neonatal sepsis. In addition to providing detectable levels of humoral immunity, it has been recently recognized that colostrum also serves to improve immune efficiency by enhancing cell-mediated immunity, as well as to provide hormones and tissue growth factors. Thus, its value to the naive neonatal foal is immense.
2.2 Interpretation of the Detection of *Sarcocystis neurona* Antibodies in the Serum of Young Horses

2.2.1 Introduction

Equine protozoal myeloencephalitis (EPM), caused by the protozoal organism *Sarcocystis neurona*, is a frequently diagnosed neurologic disorder of horses in the United States. Recent studies have estimated that 45-55% of the horse population across the United States have been exposed to *S. neurona* at some time in their lives such that they mount a measurable humoral immune response (MacKay, 1997). Exposure is determined by western blot (WB) analysis of serum obtained from horses; a positive result indicates the presence of *S. neurona* specific antibodies in the serum (Granstrom, 1995).

Evaluation of results of serologic tests in young horses for the presence of *S. neurona* specific antibodies may be complicated by several factors, including the possibility of in utero exposure and the potential for the passive transfer of *S. neurona* specific maternal antibodies. To date, it is not known whether in utero exposure to *S. neurona* occurs. Transplacental infection with protozoa is possible (Dubey, 1990), although it has not been specifically reported with *S. neurona*. The equine fetus is capable of mounting a humoral immune response at 180 days of gestation (McGuire and Crawford, 1973; Perryman et al., 1980; LeBlanc, 1990). If exposed to the organism after 180 days, specific antibodies should be present in the presuckle serum. One goal of this
study was to examine the potential for in utero exposure to *S. neurona* by determining the number of foals that have detectable serum concentrations of *S. neurona* antibodies in their serum prior to colostral ingestion.

A mare that is seropositive for EPM, and thus has circulating antibodies to *S. neurona*, would presumably pass these antibodies to her foal in the colostrum. Another goal of this study was to determine how frequently foals obtain detectable antibodies to *S. neurona* by passive transfer such that they become seropositive by WB analysis. Serial sampling of seropositive foals determines approximately when maternal antibodies are completely metabolized, rendering foals seronegative by WB. A third goal of this study was to determine time to seronegative status.

Recently, research has concentrated on the disease mechanisms of EPM, especially its propensity to affect young horses. In order for the WB to be useful, clinicians and researchers must know how to interpret the results in young horses.

### 2.2.2 Materials and Methods

*Experimental design*

Serum from pregnant mares in two locations with a high seroprevalence for *S. neurona* was collected approximately 2 months before foaling to determine serostatus. The initial sample consisted of 44 pregnant mares of various breeds.
Foalings were attended, and foals did not have access to other sources of colostrum. Samples collected immediately after birth included presuckle foal serum samples, colostrum, and mare serum samples. Postsuckle serum samples from the foals were then collected at 24 hours after birth. After at least 48 hours of age, mares and foals were placed in pastures with the broodmare herd. Exclusion from the study included those foals with inadequate passive transfer of IgG (<800 mg/dL), foals whose births were not attended, or those foals requiring medical therapy with plasma transfusions, colostrum supplementation from another mare, or any other equine biologic. The 34 foals (33 seropositive, 1 seronegative post colostrum) that met the criteria for inclusion were serially sampled at monthly intervals until at least 7-9 months of age.

**Experimental Assays**

**Radial immunodiffusion (RID)**- At 24 hours, foal serum was taken to confirm and to quantify passive transfer of maternal immunoglobulins (IgG) using a radial immunodiffusion assay kit (VMRD, Pullman WA), as described by Mancini et al (1965). Each serum sample was placed into a well of a plate of agarose containing antiserum specific for equine IgG. Following diffusion into the agarose, a ring of precipitation formed proportional to the concentration of IgG, as compared to a set of standards.

**Western immunoblot analysis (WB)**- Sera from mares and foals (presuckle, postsuckle, and serial monthly foal serum samples) were analyzed (Equine Biodiagnostics Inc.)³ for
the presence of *S. neurona* specific antibodies by WB based on the procedure developed by Granstrom et al. (1993). Briefly, *S. neurona* antigens were separated by polyacrylamide gradient gel electrophoresis. Following transfer of these antigens to nitrocellulose paper, sera were applied to the paper. Antibodies specific for *S. neurona* antigens were detected by chromagenic staining and appeared in recognizable banding patterns. Serum was primarily classified as positive or negative. A positive result was further characterized as strong, low, or weak positive.

**Statistical Analysis**

The LIFETEST procedure of the SAS system (ver. 7.01, SAS Institute, Cary, NC) was used to calculate Kaplan-Meier estimates of the mean and standard error of time to seroconversion. This utilized censored observations, including the foal seropositive at the conclusion of the study and the foal sold while seropositive.

Pearson’s correlation analysis was used to test the 24 hour serum IgG concentrations for correlation with time to seroconversion.

**2.2.3 Results**

**Serostatus of mares**

Initial screening of pregnant mares by WB analysis revealed that 89% (39/44) of the mares were seropositive for antibodies to *S. neurona*. Of the 5 original seronegative
mares, 2 had seroconverted when retested at foaling to a seropositive status, resulting in 41/44 seropositive (93%).

*Serostatus at presuckle*

There were no positive WB results of the 33 foals sampled before suckling. Interestingly, the blots were essentially blank (figure 4, middle), indicating lack of immunoglobulin reactivity in the serum as compared with those of the corresponding mares (figure 4, left), which even if seronegative, have a significant amount of background reactivity.

*Serostatus at 24 hours/post colostrum*

All 33 foals from 33 seropositive mares were positive when sampled at 24 hours of age. Therefore, passive transfer of *S. neurona* antibodies from seropositive mares occurred in 100% of the foals. WB banding patterns of the transferred maternal antibodies were identical to the mare’s serum. (figure 4, left and right)

Only 1 foal from the 3 seronegative mares survived to 24 hours of age. This foal was seronegative post colostrum.

*Serostatus through time*
As the foals were serially sampled, maternal antibody decline was evidenced by gradually decreasing immunoreactivity over time (figure 5; figure 6). In general, the length of time to negative serostatus correlated to the degree of immunoreactivity of the postsuckle sample which usually was equivalent to that of the mare. This is illustrated in figure 5 by foal A which was always negative (last tested at 9 months), foal B which showed a gradual decline to weak immunoreactivity by 5 months that persisted from 5 to 8 (not shown) months of age, and foal C which had a weaker initial immunoreactivity and became seronegative by 2 months of age. Foal D was of special interest as it exhibited the gradual decline out to 5 months but then became very positive again suggesting probable exposure by 6 months.

Thirty-one of 33 seropositive foals became seronegative by 9 months of age, with a mean time to seronegativity of 4.2 months (SE- 0.39 months) (figure 6). The range of ages to seronegative conversion was from 1 month to 9 months. Of the two remaining seropositive foals, one foal was sold at 7 months and one foal (figure 5, foal D) had not become seronegative by 9 months of age. The one foal from the only seronegative mare remained seronegative throughout the 9 month sampling period (figure 5, foal A).

**Serum IgG concentration at 24 hours and time to seroconversion**

Radial immunodiffusion of 24 hour samples showed adequate passive transfer in all of the foals (mean: 1593± 338; range: 842-2159 mg/dl IgG).
Serum IgG concentrations at 24 hours were not correlated with time to seronegativity (r= 0.099; p=0.59) (figure 7).

2.2.4 Discussion

Due to the recent growing concern over equine protozoal myeloencephalitis, horses are frequently screened for exposure to *S. neurona* by performing a WB on serum. In the young horse, several factors can complicate interpretation of a positive WB result, including the potential for in utero exposure to *S. neurona*, passive transfer of maternal antibodies to *S. neurona*, and persistence of these maternal antibodies. No previous studies have been published that characterize these factors.

The neonatal foal before suckling is fundamentally agammaglobulinemic (Jeffcott, 1975; LeBlanc, 1990). The equine placenta is characterized as a diffuse, epitheliochorial type which prevents any significant placental transmission of antibodies during gestation (Jeffcott, 1974). By 180 days of gestation, B-lymphocytes are present in the fetus and can produce and secrete immunoglobulins, primarily IgM and IgG (McGuire and Crawford, 1973; Perryman et al., 1980; LeBlanc, 1990). Small quantities of IgM (8-20 mg/dl) and occasionally trace amounts of IgG (<15 mg/dL) are detectable in the serum of newborn foals before suckling and are considered endogenous in origin (Perryman and McGuire, 1980; Kruse-Elliot and Wagner, 1984). The presence of significant quantities of IgG in serum of newborn foals before suckling is highly suggestive of in utero exposure to antigen after 180 days of gestation.
To date, it is not known whether in utero exposure to *S. neurona* occurs. Transplacental infection with the protozoan *Neospora* is commonly reported in cattle, and if fetal exposure occurs after day 150 of gestation, calves are born with a measurable humoral immune response (Anderson et al., 1994). *Neospora* organisms have been detected in an aborted fetus from a horse, indicating that transplacental infections involving protozoa are possible in horses (Dubey, 1990). Since there are a number of other biological similarities between *Sarcocystis* and *Neospora* (Marsh et al., 1996b), the possibility of transplacental infection by *Sarcocystis* also exists.

In this study, the WB of all foals sampled before suckling were essentially blank, indicating minimal presence or absence of serum antibodies. If a fetus had been exposed in utero to *S. neurona* after 180 days (Perryman et al., 1980) or if transplacental leakage of maternal antibodies had occurred, a positive WB would be expected. These presuckle samples confirm seronegativity before colostrum ingestion, allowing documentation of passive transfer.

Maternal transfer of immunity in foals occurs by ingestion and absorption of antibody-rich colostrum after birth (passive transfer). The primary immunoglobulins present in colostrum are IgG and IgG (T), the half-lives of which are approximately 20 to 23 days respectively (MacDougall, 1975; Jeffcott, 1975). Thus, maternal antibodies are present for the first months of life and are metabolized over time.

Passive transfer of *S. neurona* antibodies has been described (Fenger, 1997b), but the frequency of passive transfer and rate of metabolism have not been established. In this study, it was shown that seropositive mares do transfer antibodies against *S. neurona*
to their foals in colostrum. Antibodies to *S. neurona* were detected in all of the 33 foals born to seropositive mares post-colostrum ingestion (100%). The high rate of occurrence in this study suggests that passive transfer of *S. neurona* antibodies occurs frequently in the population at large.

The sensitivity and specificity of the WB for the detection of *S. neurona* antibodies in serum is reported to be high, approaching 90% (Cohen and MacKay, 1997). When comparing blots between a mare and her foal, the blots were extremely similar in their banding patterns. This accordance is expected, since the maternal antibodies in the foal serum are essentially the same antibodies present in the mare’s serum. This similarity also supports the repeatability of the WB. Interestingly, mares in the same pasture differed considerably in their WB banding patterns. Many of the mares have been pastured together for several years. It is unknown how long any of the mares have been seropositive, as they had not been previously screened for exposure. Additionally, it is notable that such a great percentage of the broodmare sample population was seropositive (89%), well above the reported national average (MacKay, 1997).

Kinetic studies have been performed to evaluate the decay or the metabolism rate of other specific maternal antibodies. For example, maternal antibodies to equine arteritis virus have a half life of 32 days and can remain detectable for up to 8 months (Hullinger et al., 1998). Unfortunately, antibodies to *S. neurona* cannot be specifically quantitated by current testing methods, preventing determination of a decay rate. However, mean time to seronegative conversion can be calculated, and with a large sample size, a range of times to seronegative conversion can be obtained. While not quantitative, the WB can
be interpreted in a range from positive, low positive, weak positive, to negative. As time progressed with serial sampling, in most cases, the individual blots followed this progression from positive to low to weak to negative, presumably with declining amounts of maternal antibody.

Serial sampling of the seropositive foals provided valuable information regarding the metabolism of maternal antibodies to *S. neurona*. The mean time to seronegative conversion was 4.2 months, with a wide range from 1 month to 9 months (figure 6). Unexpectedly, the foal that became negative at 9 months had a 24 hour total IgG level of 850, one of the lowest values obtained in the study. Analysis between total serum IgG at 24 hours and time to seronegative conversion showed no correlation (figure 7; r=0.099; p=0.59). This may suggest that antibody metabolism occurs at different rates in individual foals. Alternatively, it may indicate that a higher concentration of *S. neurona* antibodies is passed by some mares, not reflected in the total IgG concentration at 24 hours. Without the capacity to quantitate *S. neurona* specific antibodies in the 24 hour sample, it is difficult to draw conclusions, only to state that several foals with very high IgG concentrations at 24 hours became seronegative sooner than some foals with lower IgG concentrations.

With regard to the foal still seropositive at 9 months, it is possible that this foal was exposed to *S. neurona* in the environment and was building an endogenous immune response. She showed the expected decline in immunoreactivity with a weak positive result at 5 months and then displayed steadily increasing reactivity in the following months (figure 5, foal D).
While the seroprevalence to *S. neurona* appears to increase with age, the clinical disease of EPM appears to be a much greater risk to young horses (Saville et al., 2000b). In a retrospective study, 87.5% of clinical cases of EPM occurred in horses between 1 and 6 years of age (Boy et al., 1990). In another study of histologically confirmed cases, nearly one third were two years old or younger and more than 60% were four years old or younger, with the youngest affected horse at 2 months old (Fayer et al., 1990). Currently, young horses are being more frequently screened for exposure, especially before purchase. With the potential presence of maternal antibodies, it is difficult to interpret a seropositive test in a young horse. This study suggests that young horses cannot be accurately screened by serum testing before at least 6 months of age, as maternal antibodies may be detected. In this study, the majority of foals (87%) were seronegative by 6 months of age, suggesting the screening can be performed at 6 months. If the result is positive, the foal should be retested at 9 months.
Figure 4. *Sarcocystis neurona* western blot analyses of mares at foaling (left) and their respective foals’ presuckle (middle) and postsuckle (right) samples. Results from 10 representative animals are shown. Each set is in the same order from left to right. The arrows indicate the *S. neurona* specific immunoreactive bands.
Figure 5. *Sarcocystis neurona* western blot (WB) analyses of serial serum samples of four representative foals. Shown are the WB patterns of the mare (M), presuckle (P), postsuckle (S), and monthly sera (designated numerically as months of age) of four foals (A, B, C and D). The *S. neurona* specific immunoreactive bands are marked by the arrows.
Figure 6. Months to seronegative conversion of foals western blot seropositive to *Sarcocystis neurona* antibodies post colostrum ingestion

mean=4.2 months
Figure 7. Correlation analysis between total IgG concentration at 24 hours and months to seronegative conversion. No significant correlation is demonstrated.
Chapter 3. Cerebrospinal fluid in the Neonatal Foal

3.1 Cerebrospinal Fluid Review

3.1.1 Cerebrospinal fluid taps

Cerebrospinal fluid, a clear, colorless ultrafiltrate of plasma, bathes the CNS. Located in the ventricles of the brain and the subarachnoid space of the spinal cord, it originates from the choroid plexus and ependymal lining of the ventricles and flows caudally. The functions of CSF include suspension of the CNS tissues for protection, regulation of intracranial pressure, and maintenance of ionic and acid-base balance (Andrews, 1998).

Cerebrospinal fluid is collected for diagnostic use in neurologic disease. It can be collected from either the atlanto-occipital (AO) or lumbosacral (LS) space in the horse. The LS site can be used in the standing horse and does not require general anesthesia, which can be advantageous. The LS space is the often preferred site for collection over the AO space, as CSF flows caudally, and thus, an AO tap may not be as reflective of spinal cord disease/ inflammation (Andrews, 1998). Additionally, an AO tap requires general anesthesia which can be hazardous with a neurologic horse. Blood contamination, however, occurs less frequently with the AO tap (Mayhew, 1975).

The AO tap is performed in lateral recumbency under anesthesia. With the head flexed, the landmark for skin penetration is the intersection of the cranial borders of the atlas and the dorsal midline, with the needle aimed at the lower jaw perpendicular to the
cervical vertebrae (Mayhew, 1975). The needle advances through skin, subcutaneous tissue, fascia, dorsal atlantooccipital membrane and cervical dura mater. Cerebrospinal fluid will usually flow spontaneously in the recumbent animal.

3.1.2 Blood-Cerebrospinal Fluid Barrier and the Neonate

The blood-CSF barrier is a functional and anatomical barrier which separates the CSF from the vascular compartment of the body and performs a variety of regulatory and exclusionary functions of the body (Geiser et al., 1996). In the normal neonatal foal, CSF protein concentration is significantly higher at birth to several weeks of age compared with adult horses (Rossdale et al., 1979; Rossdale et al., 1982; Furr and Bender, 1994). Similar results were found in many species, including man (Adinolfi et al., 1976), rats (Adinolfi et al., 1976), sheep (Mollgard et al., 1977), fowl (Birge et al., 1974), and foals (Rossdale et al., 1979). Several mechanisms have been proposed to explain this higher protein level, including immaturity of the blood-CSF barrier (Adinolfi et al., 1976) and increased permeability of the blood-CSF barrier due to the birth process (Cutler et al., 1966). While it was suggested that the blood-CSF barrier is not fully developed during fetal and perinatal life (Adinolfi et al., 1976;), Saunders (1977) showed with electron microscopy that tight junctions form very early in the developing fetus.

It has been speculated that the presence of an enhanced intracellular tubular transport system in foals and other neonates allows for enhanced transport of proteins and protein fractions across the blood-CSF barrier (Rossdale et al., 1979; Rossdale et al.,
1982; Saunders, 1977). Adinolfi et al. (1976) showed that labelled IgG appeared in the CSF of 1 day old rats after injection, demonstrating that serum IgG can penetrate the blood-CSF barrier in the neonate. A recent study of normal neonatal foals showed significantly higher levels of IgG in CSF compared with adults despite similar serum values of IgG (Andrews et al., 1994). The higher IgG concentration in neonatal foals is attributed to enhanced transport of IgG from the blood across the blood-CSF barrier. Andrews (1994) suggested that this may serve as a protective mechanism in the CSF to guard against infective organisms.
3.2 Detection of antibodies to *Sarcocystis neurona* in the cerebrospinal fluid of neonatal foals

3.2.1 Introduction

Equine Protozoal Myeloencephalitis (EPM), caused by the protozoal organism *Sarcocystis neurona*, is the most commonly diagnosed neurologic disorder of horses in the United States (Dubey et al., 2001). Retrospective surveys have indicated that young horses are more susceptible to EPM (Saville et al., 2000b; Boy et al., 1990; Fayer et al., 1990). Equine protozoal myeloencephalitis is currently diagnosed by a positive western blot (WB) on cerebrospinal spinal fluid (CSF) in the presence of neurologic clinical signs. Equine protozoal myeloencephalitis has been diagnosed as early as 2 months of age (Fayer et al., 1990). Therefore, as part of a complete diagnostic workup, neurologic foals are often screened for EPM by WB.

It has been suggested that the neonatal foal, as with several other species, has a permeable blood-cerebrospinal barrier that allows leakage or transport of proteins into the CSF (Furr and Bender, 1994; Andrews et al., 1994; Rossdale et al., 1982). Previous studies have documented the presence of elevated CSF protein values and IgG concentrations in the foal compared to the adult (Andrews et al., 1994; Furr and Bender, 1994). The primary objective of this study was to determine if maternal antibodies to *S. neurona* were present in the CSF of neonatal foals post colostral ingestion. A second
objective of this study was to determine if these maternal antibodies were still present in the CSF up to 2-3 months of age which may invalidate CSF WB results in young horses.

3.2.2 Materials and Methods

Initial CSF tap

Animal Selection and Use- Fifteen healthy Thoroughbred foals between the ages of 2 and 8 days (mean 4.8 days) were selected from the Virginia Tech broodmare/foal herd maintained in Middleburg, VA. All foals were delivered naturally and all births were observed. Physical and neurologic examinations were performed prior to anesthesia. Any foal that had an abnormal delivery, an abnormal physical finding, serum IgG concentration <800mg/dl, or colostrum supplementation was excluded from the study. This study was approved by the Virginia Tech Animal Care and Use Committee.

Sample collection- Foals were anesthetized with xylazine (0.46 mg/kg; [0.21 mg/lb] IV), butorphanol (0.02 mg/kg [0.009 mg/lb] IV), and propofol (2.0 mg/kg [0.9 mg/lb] IV), placed in lateral recumbency, and maintained on propofol as needed. CSF was collected aseptically from the atlanto-occipital (AO) space as described by Mayhew (1975). Cephalic venous blood samples were obtained from the foals at the time of CSF collection for serum as were jugular venous blood samples from the mares. Cephalic venous blood samples were obtained at the time of CSF collection for serum.
**Sample analysis**- Western blot analysis (WB) for the presence of antibody against *S. neurona* was performed on foal CSF and serum and mare serum, as previously described (Granstrom et al., 1993), at a commercial laboratory. The WB results were classified as negative, weak positive, low positive, or positive.

Radial immunodiffusion was used to quantify the amount of immunoglobulin in CSF and serum, as previously described (Mancini, 1965). Serum and CSF albumin concentrations were determined spectrophotometrically. Cytology and chemistries of the CSF were also performed within 2 hours of collection.

CSF indices were calculated using the following formulas:

\[
\text{Albumin quotient} = \frac{\text{CSF albumin}}{\text{Serum albumin}} \times 100
\]

\[
\text{IgG index} = \frac{\text{CSF IgG}}{\text{Serum IgG}} \times \frac{\text{Serum albumin}}{\text{CSF albumin}}
\]

**Repeat CSF samples**

At least 7 days after the initial AO tap, a second CSF and serum sample was collected from 5 of the 13 foals previously found CSF positive. Ages ranged from 13 to 41 days. A third CSF and serum collection was performed 49 days later on the same 5 foals at which time their ages ranged from 62 to 90 days.
**Statistical Analysis**

The FREQ procedure of the SAS system (ver. 7.01, SAS Institute, Cary, NC) was used to perform an exact calculation of Pearson’s chi square to show association between the grades of serum and CSF immunoreactivity in the neonatal foal.

### 3.2.3 Results

Thirteen of the 15 mares were positive by WB for serum antibodies to *S. neurona*. The 13 foals from these mares when sampled between 2 and 7 days of age were also seropositive by WB. The 2 seronegative foals were out of the 2 seronegative mares. Twelve of the 13 seropositive foals were also CSF WB positive. There was a significant association between WB reactivity in the CSF and in the serum (P=0.0005) The amount of reactivity in the CSF was the same or one gradation lower than the serum reactivity (table 1). The 1 CSF negative, seropositive foal had only weak positive serum reactivity. The 2 seronegative foals were also CSF WB negative.

The CSF samples were consistently clean with little evidence of overt or microscopic blood contamination, as demonstrated by the low number of RBC and WBC in the samples (table 2). Mean, median, and range values were calculated for CSF albumin, CSF IgG, albumin quotient, and IgG index (table 2).

The 5 foals that were retested were still serum and CSF WB positive at the second sampling. The level of CSF immunoreactivity decreased in 3/5 foals, as did the amount of
CSF IgG. At the third sampling, 2/5 foals were CSF negative; 1/5 was seronegative. The quantity of CSF IgG was considerably decreased in all 5 samples (table 3).

3.2.4 Discussion

Retrospective surveys have indicated that young horses are more susceptible to the development of clinical EPM than older horses (Saville et al., 2000; Boy et al., 1990; Fayer et al., 1990). Specifically, young seropositive horses are more likely to have concurrent clinical signs of neurologic disease and to be WB positive for the presence of S. neurona antibodies in their CSF (Boy et al., 1990). Diagnosis of EPM is based upon clinical evidence of neurologic dysfunction and detection of antibodies to S. neurona in cerebrospinal fluid (CSF) of affected animals by WB analysis. In the presence of an intact blood-CSF barrier, antibodies detected in CSF should originate from antigenic stimulation within the central nervous system. Therefore, theoretically, antibodies present in CSF indicate the presence of the organism in the central nervous system.

The blood-CSF barrier is a functional and anatomical separation of CSF from the vascular compartment of the body. It performs a variety of regulatory and exclusionary functions (Geiser et al., 1996). It has been speculated that the presence of an enhanced intracellular tubular transport system in foals and other neonates allows for enhanced transport of proteins and protein fractions across the blood-CSF barrier (Saunders, 1977). A recent study of normal neonatal foals showed significantly higher levels of IgG in CSF compared with adults despite similar serum values of IgG (Andrews et al., 1994). In the
normal neonatal foal as with other species, CSF protein concentration is significantly higher at birth to several weeks of age in comparison to that of adult horses (Rossdale et al., 1979; Rossdale et al., 1982; Furr and Bender, 1994).

In a previous study of 33 foals, passive transfer of maternal antibodies to *S. neurona* occurred in all foals from seropositive mares (Cook et al., 2001). That study showed that foals that are seronegative to *S. neurona* at birth become seropositive within 24 hours of birth after ingestion of colostrum. With the potential for enhanced transport of serum IgG into the CSF in the neonate, this present study demonstrates that antibodies to *S. neurona* are also found in the CSF of seropositive neonatal foals from seropositive mares.

Maternal antibodies are indistinguishable from endogenous antibodies to *S. neurona* with the WB. Therefore, a positive CSF WB in a young foal may not support a diagnosis of EPM but may indicate that the foal ingested antibody-rich colostrum. The second and third CSF samplings demonstrate that antibodies to *S. neurona* can persist in the CSF for at least several months.

Increased IgG concentration in CSF can result from damage to the blood-CSF barrier, increased local production of IgG with inflammatory neurologic disease, or contamination of the CSF sample with blood during collection. As little as $10^{-3}$ µl of blood, which could correspond with as few as 8 RBC/µl of CSF, could result in a false positive WB for *S. neurona* if the blood is strongly immunoreactive (Miller et al., 1999). Performing cytology before sample submission allows inclusion of only those cases
without detectable contamination. The AO taps were performed in this study without any difficulty. Blood was not evident during any sampling which is consistent with the low RBC and WBC numbers found on cytological analysis.

Traditionally, referencing IgG concentrations in CSF and serum to albumin concentrations in CSF and serum (IgG index) can be used to better differentiate the source of the IgG (Andrews et al., 1994). Our CSF indices differed appreciably from those published by Andrews et al. (1994) using normal healthy foals of the same age range and sample size. Two factors may contribute to this disparity: an apparent difference in degree of contamination and an apparent difference in CSF-blood barrier permeability. Comparing the mean number of RBCs in our CSF samples (8 µl +/-11) to their samples (208/µl +/- 471), it is evident that the levels of contamination differ considerably. The ratios of serum IgG to CSF IgG in the 2 studies are similar (our serum: 2988 mg/dl +/- 957 to CSF 25.6 mg/dl +/- 10.8 and Andrews et al. serum: 1325 mg/dl +/- 686 to CSF 10.2 mg/dl +/- 5.5). However, our serum albumin levels were lower (mean 2007 +/- 608 vs. 2900 +/- 240), and our CSF albumin levels were nearly the same (mean 51.5 +/- 15 vs. 52 +/- 8.6). The albumin discrepancy is probably due to method differences; our albumin concentrations were determined spectrophotometrically, while theirs was determined by electrophoresis (CSF) and by chemistry analysis (serum).

Serum antibody to S. neurona determined by WB reactivity slowly declines over time and may last up to 9 months of age in some foals (Cook et al., 2001). Seronegative status occurs at a mean of 4.2 months (Cook et al., 2001). Rossdale et al. (1979) suggested that CSF protein levels decrease toward adult levels within 2 weeks of birth,
a decay that is more rapid than that illustrated by table 3 which displays the gradual
decline in CSF WB reactivity and concurrent decreasing levels of CSF IgG. This study
indicates that CSF IgG levels decline toward adult levels at several months of age.

This study demonstrates that antibodies to *S. neurona* are detectable in the CSF of
neonatal foals from seropositive mares. Because the CSF WBs were positive for the
neonatal foals and for the foals up to several months of age, this study shows that the WB
cannot be reliably employed for testing a foal less than 3 months when EPM is suspected.
Table 2. Association between grades of serum and CSF WB immunoreactivity to *Sarcocystis neurona* in neonatal foals (*P*=0.0005). Each X represents one foal.

<table>
<thead>
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<th>WB Serum</th>
<th>WB CSF</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>XX</td>
</tr>
<tr>
<td>Weak Positive</td>
<td>X</td>
</tr>
<tr>
<td>Low Positive</td>
<td>XXX</td>
</tr>
<tr>
<td>Positive</td>
<td>XXXX</td>
</tr>
<tr>
<td></td>
<td>Mean/ std</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RBC (mm$^{-3}$)</td>
<td>8 +/- 11</td>
</tr>
<tr>
<td>WBC (mm$^{-3}$)</td>
<td>0.47 +/- 0.81</td>
</tr>
<tr>
<td>CSF Albumin (mg/dl)</td>
<td>51.5 +/- 15</td>
</tr>
<tr>
<td>Albumin quotient</td>
<td>2.7 +/- 1.1</td>
</tr>
<tr>
<td>CSF IgG (mg/dl)</td>
<td>25.6 +/- 10.8</td>
</tr>
<tr>
<td>IgG index</td>
<td>0.35 +/- 0.14</td>
</tr>
</tbody>
</table>

Table 3. Summary of cytologic and CSF index values of CSF taps of neonatal foals.
<table>
<thead>
<tr>
<th>Foal</th>
<th>Age (days)</th>
<th>WB</th>
<th>CSF IgG (mg/dl)</th>
<th>Age (days)</th>
<th>WB</th>
<th>CSF IgG (mg/dl)</th>
<th>Age (days)</th>
<th>WB</th>
<th>CSF IgG (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Low</td>
<td>23.6</td>
<td>13</td>
<td>Weak</td>
<td>17.6</td>
<td>62</td>
<td>Weak</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Low</td>
<td>17.5</td>
<td>13</td>
<td>Weak</td>
<td>18.4</td>
<td>62</td>
<td>Weak</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Strong</td>
<td>22.4</td>
<td>41</td>
<td>Low</td>
<td>6.5</td>
<td>90</td>
<td>Weak</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Weak</td>
<td>35.6</td>
<td>34</td>
<td>Weak</td>
<td>12.1</td>
<td>83</td>
<td>Negative</td>
<td>8.4</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>Low</td>
<td>15.0</td>
<td>35</td>
<td>Weak</td>
<td>2.4</td>
<td>84</td>
<td>Negative</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Table 4. Degrees of CSF WB immunoreactivity and CSF IgG (mg/dL) in consecutive CSF taps in foals.
Chapter 4. Conclusion

These studies demonstrate that antibodies to *Sarcocystis neurona* can be detected by western blot in serum and CSF in neonatal foals of seropositive mares. Antibodies to *S. neurona* are not present in serum at birth before ingestion of colostrum and are detectable in serum by 24 hours of age after colostrum. Cerebrospinal fluid is western blot positive in neonatal foals 2-8 days of age. There is consistent banding pattern amongst the mare’s WB, postsuckle WB, and CSF WB, strongly suggesting that the antibodies in the CSF are of maternal origin.

Maternal antibodies to *S. neurona* are metabolized over time, both in serum and CSF. Repeated sampling suggests that the CSF WB is negative prior to the serum being negative. These studies suggest that the presence of maternal antibodies in young foals confounds results of the WB when used diagnostically to detect exposure to the organism or presence of the organism in the CSF. Therefore, testing of foals should be done cautiously to avoid WB false positives. Western blot positive serum cannot be accurately interpreted before 9 months of age, and WB positive CSF is questionable up to at least 90 days and potentially longer.
LITERATURE CITED


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Slebodzinski, AB, Brzezinska-Slebodzinski E., Nowak J., Kowalska K., 1998. Triiodothyronine (T3), insulin, and characteristics of 5’-monodeiodinase (5’ MD) in mare’s milk from parturition to 21 days post partum. Reprod Nutr Dev 38, 235-44.


**FOOTNOTES**

1 *Sarcocystis neurona* vaccine, Fort Dodge, Kansas City, Missouri.

2 Bertone JJ, McKinnon A., Groat R. Clinical field study on the use of the Snap Foal IgG. In press.

3 Jennifer Morrow, PhD. Equine Biodiagnostics Institute, Lexington, KY.
VITA

Anne Grimsley Cook, the daughter of James A. and Josette P. Grimsley III, was born in Stuttgart, Germany, and raised in Beaufort, South Carolina. She grew up an avid horse lover and competitor. She graduated summa cum laude from Sewanee, the University of the South, with an undergraduate degree in Philosophy/Religion in 1993. She attended the University of Georgia for veterinary school and graduated summa cum laude in 1997. After completing an equine medicine and surgery internship at Kansas State University, she began a residency program at the VMRCVM in the equine ambulatory section. She recently has become a diplomate of the American Board of Veterinary Practitioners in equine specialty and is completing certification in veterinary acupuncture. She is married to Dr. Wesley Cook, a small animal surgeon.