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Equine Protozoal Myeloencephalitis (EPM): Causative agent(s), Diagnosis, Clinical signs, and Current Therapeutic Approaches

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1. INTRODUCTION

Equine Protozoal Myeloencephalitis (EPM) is the most important infectious neurological disease of Western Hemisphere horses; it is caused by two apicomplexan protozoal parasites, *Sarcocystis neurona* and, less commonly, *Neospora hughesi*. EPM interferes with a horse's ability to race, work, and perform; untreated, EPM can be lethal. Location of the causative organism in the CNS is random, so clinical signs of EPM are highly variable. Any combination of neurologic signs is possible, although spinal cord involvement is the more usual presentation. Onset may be gradual or acute, the usual pattern being mild signs appearing acutely and progressing with time. Ante-mortem diagnosis of EPM is challenging, requiring careful evaluation of the animal's history, clinical signs and laboratory data with rigorous exclusion of other causes. Definitive diagnosis of EPM is dependent on necropsy demonstration of typical CNS lesions of the disease and or presence of the appropriate causative organisms.

While careful clinical examination remains the most important ante-mortem diagnostic tool for EPM, laboratory methods have been developed to assist clinical diagnosis. As such, animals with clinical signs consistent with EPM should optimally have immunoblot or ELISA analyses performed on blood and cerebrospinal fluid (CSF) samples prior to diagnosis and initiation of treatment.

Preventative approaches to EPM are not well defined. Prevention of EPM with daily pyrantel tartrate (Strongid®C)[1] administration at the current labeled dose has not been shown to be effective in immunocompetent horses (Rossano *et al.*, 2005) and similarly in IFN-gamma knockout mice (Lindsay and Dubey, 2001), even though the compound is active against *S. neurona in vitro* (Kruttlin *et al.*, 2001). Recently, an EPM vaccine based on homogenates of *S. neurona* merozoites with conditional licensure has been marketed e for prevention of EPM, but the efficacy of this vaccination has not been established by prospective studies .

Therapeutic approaches to EPM are evolving. The first approaches were based on the classic anti-malarial pyrimethamine/sulfonamide combinations; this approach requires several months of therapy and has a relatively high relapse rate. Research in the 1990s identified the therapeutic efficacy of diclazuril and related agents; in 2001 toltrazuril sulfone, Ponazuril (Marquis®)[2] became the first FDA approved treatment for EPM. Soon thereafter, Nitazoxanide (Navigator™) (2003) was FDA approved for EPM, but has since been withdrawn. A pyrimethamine/sulfonamide combination formulation (ReBalance™)[3] received FDA approval in 2004 for the treatment of EPM. Additionally, Protazil[4] (1.56% diclazuril), a topical feed dressing formulation of diclazuril for use in the prevention and treatment of EPM has received FDA approval (2007) and is on the point of market release.

An important pharmacokinetic characteristic of the clazuril medications is their relatively long plasma half-lives, varying from two and four days depending on the specific medication. Because it takes approximately four plasma half-lives to attain steady state drug concentrations in plasma and CSF, the use of loading doses for these agents should be

considered, particularly in acute clinical situations. Reasonable loading dose approximations include a 2X dose on the first and/or second day of treatment, followed thereafter by the manufacturers recommended daily dose schedule. These loading dose schedules greatly accelerate the attainment of therapeutic blood and CSF levels and onset of the therapeutic response; as such they are associated with essentially no risk and much potential benefit, most particularly in acute clinical situations. The ideal therapeutic agent for use against EPM would be an orally effective agent with high efficacy against *S. neurona* and minimal toxicity for horses, an agent which would allow considerable latitude in dosing regimen without running undue risk of toxic or adverse effect. This article reviews the current information available for EPM including causative agent(s), diagnosis and clinical pharmacology/efficacy of FDA approved and non-approved investigational medications for the treatment and/or prophylaxis of EPM.

2. HISTORYOF EPM :

The disease now known as Equine Protozoal Myeloencephalitis, EPM, was first discussed at the American Association of Equine Practitioners annual convention in 1968 in Philadelphia (Rooney *et al.*, 1970). Forty-four cases of what came to be known as EPM had been identified by researchers at the University of Kentucky in the 1960s (Prickett, 1968). The disease was first called "segmental myelitis" and later changed to "focal myelitis-encephalitis" to reflect focal or small scattered areas of damage in both the spinal cord and brain. The causative agent was thought to be a fungal toxin from moldy feed due to the apparent high summer incidence of EPM. . On the other hand, the microscopic lesions were different from those found in moldy corn poisoning. The causative protozoa were not identified in the lesions until 1974 (Beech & Dodd, 1974; Cusick *et al.*, 1974; Dubey *et al.*, 1974). The etiology, diagnosis and prevalence of EPM therefore remained unknown for almost two decades after the disease was first described.

The causative parasites were first isolated in bovine monocyte cell culture from the spinal cord of a New York horse. It was named S*arcocystis neurona*, a member of the phylum apicomplexa, and was identified as the cause of the neurologic disease now known as EPM (Dubey *et al.*, 1991). More recently, a similar neurologic disease has been described in horses from which *Neospora* species have been isolated (Daft *et al.*, 1997; Marsh *et al.*, 1998; Hamir *et al.*, 1998, Finno *et al.*, 2007; Finno *et al.*, 2010). Even though the organism is recognized by antibodies against *N. caninum*, it has been reported that this organism represents a new species, *N. hughesi*, and differences in the amino acid sequence of two immunodominant surface antigens (NcSAG1 and NcSRS2) support suggestions that this organism is a new etiologic agent in equines (Marsh *et al.*, 1999). An indirect fluorescent antibody test (IFAT) for *N. hughesi* antibodies has been validated by testing serum and CSF collected before and after experimental infection with *N. hughesi* with a cut-off titer in serum of 1:640 and a cut-off in the CSF of 1:5 (Packham *et al.*, 2002). Limited studies have been performed to measure the prevalence of exposure to *N. caninum* or *N. hughesi*, both causative agents of EPM. Serologic investigations in two surveys in the US have indicated that 23.3% (Dubey *et al.*, 1999) and

11.5% (Cheadle *et al.*, 1999) of horses have antibodies reactive with *N. caninum*. One serologic investigation reported Neospora agglutination test antibodies in 55% of 67 horses from France (Pronost *et al.*, 1999). It is clear that additional studies are required to establish the importance of *Neospora* species as etiologic agents of neurologic disease in horses.

LIFE CYCLE OF S. neurona:

The life cycle of *S. neurona* is not completely known, although its life cycle can be approximated. Species of *Sarcocystis* invariably use two hosts for completion of their life cycles (Odening, 1998). The definitive hosts [sexual (gametogony) and asexual (sporogony) development] for *Sarcocystis* are predator or scavenger animals and the intermediate hosts (asexual (schizogony and merogony) development) are usually prey animals. The results of several studies have indicated that the possible definitive hosts for *S. neurona* are the opossums, *Didelphis virginiana and Didelphis albiventris*. Additionally, the range of the opossum in North America corresponds well to the geographical distribution of EPM cases. Beyond this, EPM-like diseases have been identified in a variety of animals species including cats, mink, raccoons, skunks, pacific harbor seals, ponies, and southern sea otters.

Sarcocysts of *S. neurona* have not been found in the nervous or muscle tissues of infected horses, only schizont and merozoite stages have been seen in the CNS of horses and in cultured cells (Dubey *et al.*, 1991). Horses, therefore, are considered aberrant or dead-end hosts for *S. neurona*. On the other hand, a recent study showed the presence of schizonts in the brain and spinal cord and mature sarcocysts in the tongue and skeletal muscle of a 4-month old filly with neurological disease consistent with EPM suggesting that horses have potential to act as intermediate hosts (Mullaney *et al.*, 2005). The mechanism of entry of merozoites into the CNS is not known, but is has been suggested that the parasite is transported to the CNS probably on or in leukocytes (Dubey 2001).

CLINICAL PRESENTATIONS OF EPM:

EPM is, therefore, an infectious neurological disease of American horses affecting the central nervous system (CNS). Infection with this disease can interfere with horse's ability to race, work, and perform in equestrian events (Granstrom *et al.*, 1993). Since the disease can affect both the brain and spinal cord of horses, any combination of neurologic signs are possible (MacKay, 1997a), although spinal cord involvement is more common than brain disease. Onset of signs can be gradual or rapid, but mild signs often appear acutely and progress with time (Granstrom & Reed, 1994; Reed & Granstrom, 1993).

The parasites can affect spinal cord gray matter and/or white matter resulting in focal muscle atrophy and ataxia, respectively. Many cases of EPM start as subtle lameness which may progress to frank neurological symptoms such as ataxia, weakness, and muscle atrophy, stiffness, loss of cutaneous reflexes (Kisthardt & Lindsay, 1997). Muscle atrophy usually occurs in the gluteal and quadriceps muscles, along with gait abnormalities such as toe-dragging and stumbling (Kobluk *et al.*, 1995).

Ataxia is a classical sign of EPM and it is clinically graded on a scale of one to five. In grade five ataxia, the horse cannot stand; in grade four, the horse can be made to fall with minimal manipulation; in grade three, the horse will occasionally fall under manipulation; in grade two, a horse exhibits noticeable neurological abnormalities while walking; in grade one, the horse has minimal neurologic signs such as difficulty walking on an incline, but it is noted that an experienced horseperson may not recognize a grade one animal (Fenger, 1995).

EPM horses with spinal cord lesions may show either symmetric or asymmetric truncal and limb weakness and ataxia. These lesions may also result in demarcated areas of spontaneous sweating or loss of reflexes and cutaneous sensation (Mackay, 1997a). EPM horses with brain stem lesions can show signs of depression, asymmetric vestibular (VIII) and other cranial nerve dysfunction including masseter and temporal muscle atrophy, loss of facial sensation, drooping of the ear, upper eyelid and lower lip, head tilt, and leading to one side. Depression, weakness, difficulty swallowing, behavioral abnormality and disorientation are also signs of brain infection.

ROLE OF THE IMMUNE REPONSE:

Immunosuppression and impaired cellular immune response are believed to play a role in the progression of EPM. On the other hand, these observations are commonly associated with studies of other parasites including *Toxoplasma gondii* (Alexander *et al.*, 1997) and *Neospora caninum* (Baszler *et al.*, 1999). The resistance to infection in rodents against these two parasites is regulated mainly by cellular immune response and cytokines such as interferon-gamma and interleukin 12 are important mediators of this resistance. There are, however, no specific studies conducted on the role of cellular immune response against *S. neurona* in horses.

Recently, the role of immune responses against *S. neurona* in the progression of multifocal encephalitis in mice has been studied (Marsh *et al.*, 1997). In this study, it was shown that methylprednisolone acetate (6 mg) treated C57BL/6 mice were very resistant to infection with *S. neurona.* Multifocal encephalitis was induced by *S. neurona* in C57BL/6 nude (lacking normal B- and T- cells) (congenic) mice. In a similar study, multifocal encephalitis was induced in interferon-gamma "knock-out" mice (BALB/c-Ifng^{tm1Ts}) by subcutaneous injection of SN-2 and SN-3 isolate merozoites or by oral administration of sporocysts from opossums (Dubey & Lindsay, 1998).

EXPERIMENTAL MODELS OF EPM

Experimental infection studies of EPM in horses are limited, largely t because it is very difficult (and expensive) to generate EPM experimentally (Fenger *et al.*, 1997a). In one study five foals negative for antibodies to *S. neurona* were given one to three doses of sporocysts (Fenger *et al.*, 1997a). All foals seroconverted 19 to 42 days after the first dose, and antibodies were first present in the CSF at 28 to 42 days after challenge. At necropsy, no gross

lesions were found in the CNS and, also no protozoa were detected in histological examination of CNS. Similar results were obtained by researchers in Florida and Ohio (Cutler *et al.*, 2001; Saville *et al.*, 2001). In these studies, it has been shown that clinical signs were more severe in horses given multiple doses of sporocysts and in those that were stressed by transportation. The transport stress model for the experimental induction of EPM has also been used in other studies (Saville *et al.*, 2004; Sofaly *et al.*, 2004; Furr *et al.*, 2006).

In another l EPM induction study, living merozoites of SN2 isolate of *S. neurona* were injected in the subarachnoid space of five horses which were negative for antibodies to *S. neurona* in their serum and CSF (Lindsay *et al.*, 2000a). None of the horses developed clinical signs over a 132-day observation period, but all the horses were seropositive for *S. neurona* in their CSF and serum samples at 3-4 weeks post-infection. Two horses from this group were examined at necropsy and parasite specific lesions were not identified in their tissues and also parasites were not recovered from portions of their spinal cords inoculated on to cell cultures (Lindsay *et al.*, 2000a).

Since it is believed that immunosuppression and stress are involved in the progression of EPM, researchers recently used immunosuppressive agents in order to induce EPM experimentally in the horse (Cutler *et al.*, 2001). In one of these studies, eight seronegative horses for *S. neurona* were given sporocysts for 7 days, and horses were examined for abnormal clinical signs, blood and CSF samples were collected at intervals for 90 days. Four of the challenged horses were given dexamethasone (0.1 mg/kg orally once daily) for the duration of the experiment. All the challenged horses' immunoconverted against *S. neurona* in blood within 32 days of challenge and in CSF within 61 days, and the dexamethasone treated horse's immunoconverted earlier than the control horses. At necropsy, mild to moderate multifocal gliosis and neurophagia were found in the spinal cords of 7/8 challenged horses. On the other hand, no organisms were seen either in routinely processed sections or by immunohistochemistry. Additionally, immunosuppression by dexamethasone did not increase the severity of clinical signs or the histological lesions in the CNS (Cutler *et al.*, 2001).

In summary, it is clear that some of the experimental challenges induced typical clinical signs of EPM, but the parasite was not demonstrated in any equine tissues, suggesting that healthy horses are readily capable of eliminating *S. neurona* from their tissues. Overall, healthy horses appear to be quite resistant to experimental challenge with *S. neurona* and horses successfully cleared the parasite from the CNS in most cases. Therefore, the role and mechanism of immune response in horses require further studies to determine their effects on the progression of EPM.

3. DIAGNOSIS

3.A. PHYSICAL AND NEUROLOGIC EXAMINATIONS

EPM is the most widespread and important protozoal disease afflicting horses in the Americas. Horses diagnosed with EPM include all breeds and genders and are from all backgrounds. Despite the fact that there are numerous laboratory tests for the diagnosis of

EPM, the clinical diagnosis of this disease is very challenging. Accurate clinical diagnosis of EPM can only be achieved by careful interpretations of the animal's history, the results of physical and neurological examinations, ancillary diagnosis, and laboratory findings.

The clinical signs of EPM can theoretically mimic those of any CNS disease, because infection of any part of the CNS is possible. EPM lesions in the CNS are usually focal and circumscribed resulting in generally asymmetric clinical signs. (MacKay *et al.*, 1992). Ataxia is one of the most common clinical signs of EPM and it is important to include EPM in the list of differential diagnoses for equine neurologic or musculoskeletal diseases (Kisthardt & Lindsay, 1997).

Differential diagnosis of horses with EPM showing clinical signs of ataxia or paresis should include cervical vertebral instability/stenosis (wobbler syndrome), equine degenerative myeloencephalopathy, equine herpes 1 neuritis/vasculitis myeloencephalopathy, and trauma (Bentz *et al.*, 1999). Differential diagnosis of horses with cranial nerve deficits without cerebral signs should include cauda equina syndrome, guttural pouch disease, otitis media/interna, and cranial trauma as well as EPM. Additionally, EPM horses with cerebral signs (depression) with cranial nerve deficits or ataxia may present similarly to horses with viral encephalitides, leukoencephalomalacia, hepatoencephalopathy, or rabies (Bentz *et al.*, 1999).

Careful evaluation of the patient's history and symptoms are very important for the diagnosis of EPM. The neurologic examination should include complete cranial nerve evaluation, evaluation of conscious and unconscious proprioception (sense of limb position), conscious sensation of noxious stimuli, simple reflex evaluation, and evaluation of the moving animal for gait changes indicative of a neurologic deficit. Ambiguous or nonspecific findings such as lameness, weakness, loss of condition, uncharacterized muscle atrophy or wasting and subtle gait changes are not specifically diagnostic of neurologic deficits. Therefore, these symptoms may not be used for definitive diagnosis of any specific neurologic disease such as EPM (Bentz *et al.*, 1999).

Gait aberrations occurring with EPM vary greatly in severity and neuroanatomic location. Numerous non-neurologic processes can cause mild gait changes, therefore gait evaluation should be performed with all clinically compatible neurologic and non-neurologic conditions in mind. Cranial nerve deficits are often indiscriminately associated with EPM; for example many instances of facial nerve deficits without other neurologic abnormalities are most commonly due to direct trauma of the facial nerve (Bentz *et al.*, 1999).

Horses that display signs of vestibulopathy (CN VIII dysfunction) should be evaluated in order to localize the lesion as central or peripheral. Peripheral vestibular disease should always be considered in horses with vestibulopathy without other spinal cord neurologic deficits (Bentz *et al.*, 1999). Facial nerve deficits that are localized outside of the brainstem can be associated with peripheral vestibular disease and vestibular disease cannot be considered a specific finding of EPM. Despite a description of an association of EPM with a "brainstem

syndrome" involving the seventh and eighth cranial nerves (MacKay 1997a), there is neither a physiologic rationale nor pathologic evidence that *S. neurona* exhibits a specific affinity for any cranial nerve nucleus. Therefore, radiography, otoscopy, endoscopy and/or scintigraphy of the skull for peripheral vestibular disease are indicated as components of the complete evaluation of horses exhibiting vestibulopathy (Bentz & Ross, 1997).

Muscle wasting due to prolonged illness is usually mistaken as neurologic muscle atrophy. Incidence of muscle atrophy related to neurologic lesions occurs less diffusely compared to muscle wasting associated with prolonged illness. Muscle atrophy is highly associated with EPM, but it should be kept in mind that any focal neurologic disorder may cause muscle atrophy. Muscle atrophy is not usually obvious with spinal cord disease due to EPM (MacKay, 1997b). [Levant, is this statement correct? In my opinion one of the classic signs of EPM is asymmetric muscle atrophy]

Finally, the conclusions from neurologic examinations often differ among equally regarded clinicians, leading to disagreement as to what is defined as a "neurologic deficit". Indeed, the neurologic examination itself exhibits its own "clinician-specific" sensitivity and specificity. In the assessment of neurologic examination findings, clinicians that tend to identify more subtle signs as neurologic deficits are likely to have a very high sensitivity in the clinical identification of EPM. However, based on the generally accepted low disease prevalence, he/she will inevitably misdiagnose many horses as having EPM (low specificity). Animals that are misdiagnosed with EPM and that receive CSF immunoblot analysis will yield a preponderance of false positive test results (Bentz *et al.*, 1999).

In clinical evaluations of EPM, it is important to realize that the incidence of musculoskeletal disease (lameness) is much higher than that of EPM. There is no study confirming EPM as a possible cause of primary lameness. Another clinical finding associated with EPM is back pain (MacKay, 1997a). However, back pain is, again, not a specific finding for EPM, because any type of lameness altering a horse's gait can lead to secondary back pain.

3.B. LABORATORY AND ANCILLARY DIAGNOSIS

Definitive diagnosis of EPM by history and clinical signs alone is, in most cases, not possible. Therefore, numerous laboratory methods have been developed to assist in the ante-mortem diagnosis of EPM. Post-mortem diagnosis is either achieved by the demonstration of protozoa in CNS, and/or by identifying the characteristic histopathological changes when the organism is not seen in CNS (Fayer *et al.*, 1990). The usage of *S. neurona* merozoites as an antigen in indirect immunofluorescent antibody (IFA) test was not successful, because of cross-reactivity of antibodies with other equine *Sarcocystis* species and with *S. neurona* merozoites (Granstrom *et al.*, 1993). On the other hand, a recent immunofluorescent assay [IFA] test has shown good diagnostic efficiency for EPM, and it was suggested that the IFA test is more sensitive and specific than the immunoblot, and the results are not influenced by blood contamination, unlike immunoblot analysis (Duarte *et al.*, 2004; Duarte *et al.*, 2006).

One of the most important achievements in the area of ante-mortem diagnosis of EPM was the development of an immunoblot (Western blot) assay for *S. neurona* antibody (Granstrom *et al.*, 1991; Granstrom, 1993). In one of these studies, sera from seven horses with EPM and a single horse immunized with *S. neurona* merozoites exhibited specific bands in transferred sodium dodecyl sulfate- (SDS) gel electrophoretograms of *S. neurona* lysate. This immunoblot test is widely accepted as an indicator of exposure to *S. neurona* (Granstrom, 1993). It has been suggested that animals with clinical signs consistent with multifocal central nervous system lesions should have immunoblot analyses performed on blood and CSF.

In a recent study, it has been reported that *S. neurona* isolates (four from California and one from Missouri) from different geographic regions have distinct phenotypic patterns (Marsh *et al.*, 2000). In this study, monoclonal antibodies were produced against the *S. neurona* isolate (UCD-1). The monoclonal antibody 2A7 reacted to a single protein band by Western blot analysis to all the California isolates but not against the isolate from Missouri. Therefore, it was concluded that all the parasites studied were *S. neurona*, but the *S. neurona* isolate from Missouri was antigenically distinguishable from other strains compared. The authors suggested that it would be important to consider host responses to antigenic variations and possible surface proteins differences when evaluating potential diagnostic or immunizing parasite proteins.

A recent study suggested that relatively minor changes in developed immunoblot tests might increase the test sensitivity and specificity to nearly 100% (Rossano *et al.*, 2000). The Western blot test was initially developed to detect antibodies to *S. neurona*-specific antigens (approximately 22.5, 13, and 10.5 kD) in serum and cerebrospinal fluid of horses suspected to have EPM (Granstrom *et al.*, 1993). Additionally, a study indicated that immunodominant proteins of 29 and 17 kD are specific to *S. neurona*, therefore, setting new criteria for a positive EPM test (Lapointe *et al.*, 1998). This study showed that the *S. cruzi* antibody-blocked Western blot, obtained by treating blots with bovine *Sarcocystis cruzi* antibodies prior to loading the equine samples, had a sample sensitivity of 100% and sample specificity of 98% (Rossano *et al.*, 2000). In this study, it was shown that the specificity of the Western blot test could be improved by blocking proteins not specific to *S. neurona* and using reactivity to the 30- and 16-kD bands as the criterion for a positive test. This study also suggested that bovine *S. cruzi* antibodies bind to *Sarcocystis* genus-specific proteins but not to *S. neurona* specific proteins. Therefore, by blocking the nonspecific proteins that may be too close to distinguish from the specific protein, the Western blot test could be improved. On the other hand, it is known that *S. cruzi* does not infect the horse and horses are not known naturally to produce antibodies to the parasite. Additionally, the original Western blot test was developed by excluding *S. neurona* proteins that cross-reacted with *S. cruzi*, *S. muris*, or *S. fayeri* antisera. Therefore, further studies need to be done to confirm that the *S. cruzi* antibody-blocked Western blot test can increase test sensitivity and specificity to approximately 100%.

Serum immunoblot tests are generally run at 1:10 dilution, and a positive test is indicated by reactivity with the specific bands. However, the serum immunoblot test only indicates exposure to the parasite (Blythe *et al.*, 1997; Saville *et al.*, 1997), and therefore, the

CSF immunoblot analysis can be an important diagnostic aid (Bentz *et al.*, 1997; Saville *et al.*, 1997). Additionally, immunoblot testing of 1:1 diluted CSF samples with Blotto/Tween-20 compared to the serum test apparently has much greater specificity for EPM. CSF immunoblot has been shown to exhibit 89% sensitivity and specificity as defined by the presence or absence of the post-mortem EPM lesions in the CNS (Granstrom, 1997).

The positive predictive value (PPV; the proportion of truly diseased animals among all those tested positive) calculated from CSF test results was 85%. The negative predictive value (NPV; the proportion of non-diseased animals among all those that tested negative) for CSF results was 92%. Additionally, it was suggested that CSF testing should be performed for the diagnosis of EPM among horses with neurologic symptoms, and it is not useful to screen CSF from normal horses (Granstrom, 1997, Cohen & MacKay, 1997). Beyond this, it was suggested that the negative CSF immunoblot test from horses with clinical signs of EPM (false-negative) in acute cases should be repeated at a later date to allow production of detectable amounts of antibody. Protozoa were seen in 7 of 70 horses with presumptive EPM diagnoses, indicating difficulties in the histological diagnosis in both the positive and the negative groups.

In another recent study, sensitivity and specificity of CSF immunoblot test were determined in 234 horses with EPM, as defined by the presence or absence of the post-mortem lesions in CNS (Daft *et al.*, 2000). In this study, based on pathologic findings in the CNS, horses were classified as EPM-negative, EPM-positive (*S. neurona* demonstrated in lesions by immunostaining) and EPM-suspect (specific EPM lesions present but *S. neurona* not demonstrated). EPM-positive and EPM-suspect horses were combined for analysis. Of 112 horses that tested CSF Western blot positive, post-mortem exam of nervous system tissue showed only 12 horses as actually EPM-positive. Eight and 92 horses in this group were EPM-suspect and EPM-negative, respectively. The CSF-negative group (n=122) consisted of 3 EPM-suspect and 119 EPM-negative horses according to post-mortem results.

For all horses combined, sensitivity and specificity of the CSF Western blot test were 87% and 56%, respectively. When the results for normal (n=169) and neurologic horses (n=65) were analyzed separately, the sensitivity was similar for both groups, and specificity was 44% for neurologic and 60% for normal horses, indicating 56% and 40% of CSF Western blot tests were false-positive for each group, respectively. For normal and neurologic horses, the predictive value for CSF-negative horses was 99% and 92% whereas for CSF-positive horses it was 10% and 32%, respectively. Since CSF Western blot test results when negative had accuracy more than 90% for both neurologic and normal horses, this study suggested that CSF Western blot negative test is very useful in ruling out EPM. This study suggested that Western blot test results should be interpreted very cautiously, and identified the clinical examination procedure as the most useful ante-mortem diagnostic tools for diagnosing EPM.

The presence of anti-*S. neurona* antibody found within CSF of non-EPM horses (false-positive immunoblot tests) might be associated with various circumstances such as contamination of CSF samples with the serum *S. neurona* antibody (MacKay, 1997a; Andrews *et al.*, 1990). Additionally, breakdown of the blood-brain barrier due to disease/inflammation

unrelated to EPM (e.g., equid herpesvirus-1 myeloencephalitis, trauma) may cause false-positive test results. False-positive tests can also be due to filtration of antibody from blood containing high levels of antibody into the CSF at levels detectable by immunoblot analysis, persistence of antibody in CSF for long periods after clearance of the organism, or presence of the organism within the spinal cord without clinical signs of EPM (Bentz *et al.*, 1999). Any CSF sample that is discolored or has a red blood cell (RBCs) count greater than 500/µl should be checked by albumin quotient (AQ) and immunoglobulin G index (IgGI) (Green *et al.*, 1993).

On the other hand, there are also concerns about how to interpret positive CSF samples from clinically normal horses; ruling out subclinical EPM infections in these cases is difficult. CSF positive samples from neurologically normal horses are often called "false-positives" because they are not predictive of clinical disease (Rossano *et al.*, 2000). It has been suggested that further studies will be necessary to determine the association of CSF antibodies to *S. neurona* with the actual presence of the parasite and, similarly, the association of the presence of *S. neurona* with clinical cases of EPM (Rossano *et al.*, 2000).

In normal horses, the concentration of protein in CSF is less than 100 mg/dl and the normal cell count is fewer than 5 cells/µl (Green *et al.*, 1992; Green *et al.*, 1993). Ideally, a CSF sample should have < 5 RBCs/ µl, but CSF samples with up to 50 RBCs/µl can still be used for immunoblot testing with carefully interpretation of the data (Furr *et al.*, 2002). The CSF albumin quotient (AQ) and IgGI [ImmunoglobulinG index] have been used to determine the integrity of the blood-brain barrier and intrathecal immunoglobulin production (Andrews *et al.*, 1995). Albumin, the most abundant serum protein, is not produced in the CSF and it must enter the CSF from serum. Although the AQ is considered to be a useful parameter in determination of serum protein contamination of the CSF, the IgGI has received less enthusiastic support as a useful diagnostic parameter (Cohen & MacKay, 1997; Miller & Bernard, 1996). The AQ is the ratio of the concentration of albumin in CSF to that in serum multiplied by 100. An AQ greater than 2.03 suggests the presence of serum protein in the CSF sample as a result of sample contamination with blood or increased permeability of the blood-brain barrier.

The IgGI is the ratio of the concentration of IgG in CSF to that of serum, divided by the AQ. The IgGI test is used to evaluate the increased intrathecal productions of IgG and to differentiate increased IgG level in CSF (due to leakage or contamination from blood) from increased intrathecal production of IgG. Therefore, an IgG index greater than 0.3 suggests intrathecal IgG production and it is believed that this IgG is the source for positive immunoblot. The albumin quotient should be combined with a measure of the IgGI. However, some researchers believe that the IgGI provides limited diagnostic information regarding diagnosis of EPM, but that it may provide useful information regarding response to treatment (Furr *et al.*, 2002).

Currently, Enzyme Linked Immunosorbent Assay (ELISA) is also used for the diagnosis of EPM (Hoane *et al.*, 2005; Heskett & Mackay, 2008; Yeargan & Howe, 2011; Furr *et al.*, 2011). One ELISA is commercially available from Pathogenes Inc. (Fairfield, FL, USA)

and the second is available from Equine Diagnostic Solutions, LLC (Lexington, KY, USA). In a recent study, it has been shown that Goldman-Witmer coefficients (*C*-values) and the antibody index (AI) are very useful in the diagnosis of EPM (Furr *et al.*, 2011). In this study, *C*-values were calculated as the reciprocal CSF titer times the total serum IgG divided by the total CSF IgG times the reciprocal serum titer. The AI was calculated as the ratio of the specific antibody quotient (QAb) over the equine albumin quotient ($QAlb_e$). In this study, serum and CSF samples from patients with either EPM or cervical vertebral compressive myelopathy (CVM) were tested using an ELISA against the SnSAG2protein. Sensitivity and specificity for AI was 71 and 100% and for *C*-value was 86 and 100%, respectively. The results of this study demonstrated the value of calculating the AI and *C*-value in the diagnosis of EPM, even in the presence of red blood cell contamination (Furr *et al.*, 2011). In a related study, it has been shown that serologic diagnosis of EPM can be improved by modifying the existing ELISAs based on the immunogenic parasite surface antigens SnSAG2, SnSAG3, and SnSAG4 to make the assay polyvalent (Yeargan & Howe, 2011). The results of this study suggested that the use of polyvalent SnSAG ELISAs will enhance the reliability of serologic testing for *S. neurona* infection, thereby improving the accuracy of EPM diagnosis. (Yeargan & Howe, 2011)

A polymerase chain reaction (PCR) test was also developed to evaluate the presence of *S. neurona* DNA in CSF, and was suggested to confirm negative or suspect Western blot test results (Fenger *et al.*, 1995). However, it is now believed that the PCR test is a low yield and low sensitivity test (Bernard, 1997; MacKay, 1997a, Miller & Bernard, 1996) because of the not uncommon absence of either the DNA or the organism in CSF samples. It has therefore been recommended that the PCR test should not be used alone as a diagnostic tool for EPM, but rather in combination with the Western blot test (Miller & Bernard, 1996). In this study, it was found that 106 horses with positive CSF Western blots tested negative with PCR, indicating low probability of obtaining a PCR positive in the presence of an intrathecal antibody response. This study also recommended the use of PCR tests in identification of EPM in the few infected horses that are CSF Western blot negative. In most of the acute cases enzymes released during the host's response to infection can destroy the parasite DNA, thereby yielding false negative PCR test results (Marsh *et al.*, 1996). Additionally, since CSF Western blot positive horses are infrequently PCR positive, and a small percentage of CSF Western blot negative horses may be CSF PCR positive, clinical use of the PCR test is not recommended. (Miller & Bernard, 1996).

4. CURRENT THERAPEUTIC APPROACHES FOR EPM.

4.A. THE ROLE OF PYRIMETHAMINE/SULFONAMIDE COMBINATIONS IN TREATMENT OF EPM:

Current treatments for EPM include co-administration of sulfonamides (sulfadiazine or sulfamethoxazole) and pyrimethamine, a protocol developed for use as anti-malarial therapy. Sulfonamides act by competing with para-aminobenzoic acid (PABA) as substrate for the

enzyme dihydropteroate synthase, which incorporates PABA into dihydropteroic acid, the immediate precursor of folic acid (Krungkrai *et al.*, 1989). Figure 1 shows the structures of PABA and various sulfonamides. In keeping with this approach, Phoenix Scientific® has recently brought to market an FDA-approved formulation of pyrimethamine and sulfadiazine, marketed as ReBalance™[3]. ReBalance™ antiprotozoal oral suspension is supplied in 946.4 mL (1 qt) bottles. Each ml of ReBalance™ antiprotozoal oral suspension contains 250 mg sulfadiazine as the sodium salt and 12.5 mg pyrimethamine. ReBalance™ oral suspension is to be administered at a dose of 20 mg/kg sulfadiazine and 1 mg/kg pyrimethamine daily or 4 ml of ReBalance™ suspension per 110 lb (50 kg) of body weight once per day. The duration of treatment is dependent upon clinical response, but the usual treatment regimen ranges from 90 to 270 days.

Trimethoprim and pyrimethamine inhibit dihydrofolate reductase activity necessary for purine and pyrimidine nucleotide synthesis (Kisthardt & Lindsay, 1997). Figure 2 shows the structures of pyrimethamine and trimethoprim. Dihydropteroate synthase is not present in mammalian cells, while pyrimethamine and trimethoprim are more active against the parasite's dihydrofolate reductase than against the mammalian enzyme (Kisthardt & Lindsay, 1997). Therefore, combination therapy synergistically interferes with the folic acid metabolism and biosynthesis of purine and pyrimidine nucleotides that are necessary for the parasite's survival and multiplication. Since pyrimethamine is more active than trimethoprim *in vitro* against *S. neurona*, this compound is usually included in EPM treatment (Lindsay & Dubey, 1999). Figure 3 summarizes the mechanisms of action of trimethoprim, pyrimethamine and the sulfonamides.

The recommended combination therapy for EPM includes the use of liquid formulation of sulfadiazine 20 mg/kg and pyrimethamine 1 mg/kg orally s.i.d. or trimethoprim/sulfonamide 15-20 mg/kg orally b.i.d. combined with pyrimethamine 1 mg/kg b.i.d. for 30 days after clinical signs have stopped improving (Kisthardt & Lindsay, 1997). It has been shown that pyrimethamine is completely coccidiocidal at 1 μg/ml with predicted IC_{50} (inhibitory concentration) of 0.5 μg/ml (Lindsay & Dubey, 1999). No regrowth of parasites occurred in cell cultures treated with 1 or 5 μg/ml pyrimethamine. Trimethoprim was completely inhibitory and coccidiocidal at 5 μg/ml with predicted IC_{50} of 2.5 μg/ml. None of the six sulfonamides examined at 50 or 100 μg/ml had activity against *S. neurona* in microtiter assay. On the other hand, it was shown that *in vitro* combination of 0.1 μg/ml pyrimethamine with 5 or 10 μg/ml of sulfadiazine completely inhibited growth of *S. neurona* in bovine turbinate cell culture (Lindsay & Dubey, 1999). In this study, it was also shown that pyrimethamine and sulfonamides act synergistically against *S. neurona*; sulfonamide alone was not inhibitory and almost 10 fold more pyrimethamine alone was required for complete inhibition. The results of this study suggested that clinical relapses following EPM therapy is probably due to the failure of sustained coccidiocidal concentrations of drugs to reaching the parasites in the equine central nervous system and not due to the drug combination being only coccidiostatic (Lindsay & Dubey, 1999).

Steady state CSF concentrations of pyrimethamine (0.02-0.1 μg/ml) can be obtained

after 4-6 hours following a single oral dose of 1 mg/kg/day, indicating no requirement for a loading dose (Clarke *et al.*, 1992a). Mean steady state CSF concentrations of sulfonamide following either single or multiple doses of 22 to 44 mg/kg were found to be 2-8 μg/ml (Brown *et al.*, 1989). Additionally, it was reported that terminal elimination half-lives of pyrimethamine (Clarke *et al.*, 1992b) and sulfadiazine (Brown *et al.*, 1983) are relatively short in the horse, 12.06 hours and 2.71 hours, respectively. The relatively short plasma half-lives of these compounds suggests that there will be a large fluctuations between peak and trough concentrations of these compounds in the CSF following daily administration. Therefore, if one of the drugs is present at concentrations less than those required to produce inhibition of the pathway, the entire synergistic effect will either be lost or be greatly reduced. This means that every effort must be made to maintain full CSF concentrations of these drugs throughout the entire duration of therapy. Clarke *et al.* (1992a) also indicated that repeated doses of 1 mg/kg of pyrimethamine probably would not produce levels that exceed 0.1 μg/ml in the CSF. These results indicate that relapse of EPM is most likely due to the failure of maintenance of coccidiocidal concentrations of standard treatment drugs in the CSF due to either lack of ability of these agents to pass blood-brain barrier or to the relatively short elimination half-lives of these agents in the horse. Additionally, the optimal duration of treatment is difficult to determine, but some authorities have recommended that treatment should continue until negative CSF immunoblot results are obtained, suggesting diminution of *S. neurona* antigens. If this is correct, s standard therapy may well be required for years (Fenger *et al.*, 1997b).

Few studies have been conducted to determine the clinical efficacy of sulfonamide /pyrimethamine combination. EPM horses treated with trimethoprim-sulfadiazine have reportedly made significant improvements in neurologic signs, although two of three horses treated in one report had relapses after the medication was discontinued, suggesting incomplete removal of parasites from the CNS (MacKay *et al.*, 1992). In another combination therapy efficacy study, a 31% relapse rate was reported after the treatment was discontinued, including horses treated for varying periods, ranging from 2 weeks to over 6 months (Fenger *et al.*, 1996). In another clinical study, 102 horses treated with 0.5 or 1 mg/kg of pyrimethamine with sulfonamides for unspecified periods were reported to have a cure rate of 66% (Fenger, 1997). The treatment success with pyrimethamine/ sulfonamide combination therapy is therefore estimated to be 60-70% and the relapse rate to be 10% (Reed & Saville, 1996).

Adverse reactions associated with the standard treatment include anemia, neutropenia, thrombocytopenia, leukopenia, diarrhea and urticaria (Welsch, 1991; Clarke *et al.*, 1992a). Pyrimethamine is considered teratogenic and causes both neonatal disorders and abortion (Toribio *et al.*, 1998), and there are also suggestions that this EPM treatment can affect the breeding performance of stallions (Bedford and McDonnell, 1999). Folic acid (a precursor to dihydrofolate reductase) has been recommended to prevent toxicity associated with standard treatment in the horse (Fenger, 1997). However, folic acid is poorly absorbed in horses and has been shown to increase pyrimethamine-induced embryotoxicity in the rat model (Allen, 1984). Therefore, folic acid is currently not recommended in the treatment of EPM, and also is contraindicated in pregnant mares (MacKay, 1997a).

4.B.1. Toltrazuril Sulfone/Diclazuril/: Second generation treatments for EPM:

Diclazuril, ((+)-4chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrohydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile, toltrazuril, (1-(3-methyl-4'-trifluoromethyl-thiophenoxy)-phenyl)-3methyl-1,3,5, triazine-2, 46 (1H, 3H, 5H) trione, and toltrazuril sulfone (Ponazuril), (1-methyl-3-[3-methyl-4-(4-trifluoromethanesulfonyl-phenoxy)-phenyl]-[1,3,5] triazinane-2,6,6-trione are members of a large group of triazine-based antiprotozoal agents. The triazine ring is present in all of these compounds; the benzeneacetonitrile group (benzene ring with an acetonitrile group attaching it to the central ring structure) is present in all these compounds except toltrazuril and toltrazuril sulfone (Fleeger, 1997). Among 54 triazine analogs, diclazuril is the most potent agent against a wide variety of apicomplexans; the structures of a number of these triazine-based antiprotozoal agents are shown in figure 4.

Diclazuril is effective against intestinal *Eimeria* species in avians, intestinal and hepatic coccidiosis in rabbits, and toxoplasmosis in mice, and has broad-spectrum anticoccidial effects in many other mammalian species (Vanparijs *et al.*, 1990; Alzieu *et al.*, 1999; Lindsay *et al.*, 1995a; Lindsay & Blagburn, 1994, McDougald *et al.*, 1991). Diclazuril is fed at 1 part per million (ppm) to prevent intestinal coccidiosis in chicken (McDougald *et al.*, 1990) and also in turkeys (Vanparijs *et al.*, 1989). Diclazuril has been shown to be effective against isosporiosis, toxoplasmosis, and cryptosporidiosis in AIDS patients (Limson *et al.*, 1995; Fung & Kirschenbaum, 1996; Menichetti *et al.*, 1991, Kayembe *et al.*, 1989).

Additionally, diclazuril given orally once daily for 10 days at 1 or 10 mg/kg on day 1 prior to infection due to RH strain *T. gondii* has been shown to prevent death in 80% and 100% challenged mice (Lindsay & Blagburn, 1994). Oral treatment with 10 mg/kg diclazuril also prevented death in 90% of mice over a 56-day observation period with acute RH strain toxoplasmosis when given once daily for 10 days on day 6 when mice were clinically ill (Lindsay *et al.*, 1995b). In this study, it was also shown that diclazuril has synergistic effects with pyrimethamine, when diclazuril (1 or 5 mg/kg) was combined with pyrimethamine (12.5 mg/kg) all mice survived the 56-day observation period (Lindsay *et al.*, 1995b).

Recently, it was shown that diclazuril prevents *S. neurona* infection in gamma-interferon knockout mice at 10 mg/kg daily oral dose (Dubey *et al.*, 2001). In this study, a group of gamma-interferon knockout mice were fed a lethal dose of ~1000 sporocysts of the SN15-OP isolate of *S. neurona* and were given pelleted rodent feed containing 50 ppm of diclazuril at different times before and after feeding sporocysts. All mice were examined at necropsy and their tissues were examined immunohistochemically for *S. neurona* infection. Twenty mice were fed sporocysts and given diclazuril starting 5 days before feeding sporocysts and continuing 30-39 days post-infection, all these mice remained clinically normal and *S. neurona* organisms were not demonstrable by bioassay of the brains of these mice. *S. neurona* organisms were also not found in tissues of five mice treated with diclazuril, starting 7 days after feeding sporocysts and continuing up to 39 days post-infection. *S. neurona* organisms were found in two of 19 mice treated with diclazuril starting 10 days post-infection, in two of

five 5 starting treatment 12 days post-infection, and in 10 of 10 mice when treatment was delayed until 15 days post-infection. Six mice not fed sporocysts but given diclazuril for 44 days remained clinically normal.

The results of this study suggested that efficacy of diclazuril is lower in later stage of *S. neurona* infection in gamma-interferon knockout mice, therefore suggesting that diclazuril may be considered as a preventive rather than as therapeutic agent for EPM. On the other hand, challenged mice which had diclazuril treatment at later time points of post-infection were not treated for a similar duration compared to mice treated in earlier time point of post-infection. Additionally, since diclazuril was mixed with rodent feed and feed intake was not measured, it may be possible that the amount of feed consumed by mice with neurologic signs (treatment in later time point of post-infection) was less compared to other mice without neurologic signs (treatment in earlier time point of post-infection). Therefore, it is clear that further studies are required to determine whether or not diclazuril is more effective as a prophylactic agent rather than therapeutic agent for EPM.

It is well established that in AIDS patients pyrimethamine and trimethoprim-sulfa combination treatment not uncommonly results in development of isosporiasis. In one study (Limson *et al.*, 1995), the clinical efficacy of diclazuril was investigated in an AIDS patient infected with *Isospora belli*. Diclazuril was administered orally at 300 mg per day to one patient, and the symptoms associated with isosporiasis decreased rapidly and did not re-occur. The steady state plasma concentrations of diclazuril in this patient were 140-150 ng/ml, confirming both the oral absorption and clinical effectiveness of orally administered diclazuril. . Diclazuril has been suggested to have potential activity against *S. neurona* (Granstrom *et al.*, 1997) and preliminary data suggest that it has clinical application in the treatment of EPM (Bentz *et al.*, 1998).

Recently the efficacy of diclazuril in inhibiting merozoite production of *S. neurona* and *S. falcatula* in bovine turbinate cell cultures was examined (Lindsay & Dubey, 2000). This study has demonstrated more than 80% inhibition of merozoite production in cultures of *S. neurona* or *S. falcatula* treated with 0.0001 μg/ml diclazuril and greater than 95% inhibition of merozoite production with 0.001 μg/ml diclazuril. The merozoite production assay was conducted after 10 days of treatment of *S. neurona* infected cultures or 13 days of treatment of *S. falcatula* infected cultures. On the other hand, when diclazuril-containing medium was removed from treated flasks, renewed multiplication occurred in both *S. neurona* and *S. falcatula*-treated flasks.

In another recent study, parasite-specific incorporation of ^{3}H-uracil was used to determine the efficacy of various antiprotozoal agents against *S. neurona* in bovine turbinate cell cultures (Marsh *et al.*, 2001). In this study, it was shown that diclazuril (100 ng/ml), sulfadiazine (5 μg/ml), sulfamethoxazole (5 μg/ml), atovaquone (0.04 ng/ml) and tetracycline (5 μg/ml) treatment of infected monolayers for 5-7 days incubation period did not significantly reduce the parasite replication compared to control. Of compounds tested, pyrimethamine (0.1 to 1 μg/ml) was the most potent anti-*S. neurona* agent tested (significant reduction in *S.*

neurona replication). In this study (Marsh *et al.*, 2001), the effect of diclazuril was determined on total merozoite production (intracellular and extracellular) while in previous study (Lindsay & Dubey, 2000) diclazuril effect was determined only on free merozoites. Additionally, incubation period with diclazuril in this study was almost 50% shorter compared with the previous study. The experimental differences between these two studies may account for the different results obtained, as such; further studies are required to determine exact *in vitro* sensitivity of *S. neurona* for triazine agents and diclazuril.

Toltrazuril is effective *in vivo* against *Eimeria* species in avians, *in vitro* against *Toxoplasma gondii*, and *in vivo* against intestinal and hepatic coccidiosis in rabbits (Ricketts & Pfefferkorn, 1993; Peeters & Geeroms, 1986; Chapman, 1987, Reynaud *et al.*, 1999). Toltrazuril is principally used as an intermittent water treatment rather than as a continuous-feed medication, and it has been successfully used against a variety of protozoan parasites in fish and insects. It has been administered with the drinking water as a 2.5% water miscible solution, as a pre-mix added to feed, as an aqueous suspension for subcutaneous injection, and as a pour-on application in a 1% isopropanol solution (Vazquez & Vazquez, 1990). Additionally, this antiprotozoal agent has been shown to be effective against *S. neurona in vitro* at very low concentrations (Dr. David Granstrom, 1996, unpublished data).

The efficacy of toltrazuril sulfone, the major equine metabolite of toltrazuril, in inhibiting merozoite production of *S. neurona* in cell cultures was recently reported (Lindsay *et al.*, 1999; Lindsay *et al.*, 2000b). This study showed 94.4% and 98.5% inhibition of merozoite production in bovine turbinate cell cultures of *S. neurona* treated with 0.1 to 1 μg/ml of toltrazuril sulfone, respectively. Additionally, when *S. neurona* merozoite production assay was determined in African green monkey kidney cells (CV-1), there was 70.7% and 90.1 % inhibition of merozoite production treated with 0.1 to 1 μg/ml toltrazuril sulfone, respectively. In CV-1 cell cultures, 5 μg/ml of toltrazuril sulfone showed 97.1% inhibition of merozoite production of *S. neurona.*

The efficacy of toltrazuril and toltrazuril sulfone against experimental *Neospora caninum* infection in C57BL/6 mice has also been investigated (Gottstein *et al.*, 2001). In this study, mice were experimentally infected intraperitoneally with *N. caninum* tachyzoites and infection was monitored at three levels: clinically (symptoms), histologically (occurrence of cerebral lesions and parasites by immunohistochemistry) and at the molecular level (detection of parasite DNA by PCR). Medication was initiated 6 hours prior to infection and was maintained for 6 consecutive days. All the mice were sacrificed on day 15 post-infection. In this study, it was shown that treatment of mice with 20 mg/kg of toltrazuril or toltrazuril sulfone, applied in drinking-water formulation (2.5% diluted stock solution), completely prevented the formation of cerebral lesions in all treated animals in immunohistochemistry study. Additionally, PCR analyses of these treated animals showed that *N. caninum* DNA detection was reduced by 91% and 90% by toltrazuril and toltrazuril sulfone treatment, respectively.

The efficacy of toltrazuril sulfone against experimental *Toxoplasma gondii* infection in

CD-1 mice has recently been investigated (Mitchell *et al.*, 2004). In this study, mice were experimentally infected subcutaneously with *T. gondii* tachyzoites and infection was monitored serologically (measuring antibodies using direct agglutination test), and on the molecular level (detection of parasite DNA by PCR). Medication was initiated either 1 day before infection using 10 or 20 mg/kg toltrazuril sulfone, 3 days after infection using 10 or 20 mg/kg toltrazuril sulfone, or 6 days after infection using 10, 20 or 50 mg/kg of toltrazuril sulfone and the medication was administered orally in a suspension daily for 10 days regardless of the dose and onset of treatment. In this study, it was shown that treatment of mice with either 10 or 20 mg/kg of toltrazuril sulfone 1 day before infection completely protected against acute toxoplasmosis and relapse did not occur after prophylactic treatments were stopped. Treatment of clinical toxoplasmosis at 6 days after infection was less effective with 10 mg/kg (60%) survival) than with 20 or 50 mg/kg (100% survival). This study also showed that toltrazuril sulfone at 5, 1, and 0.1 μg/ml significantly inhibited *T. gondii* tachyzoites production in African green monkey kidney cells. Even though toltrazuril sulfone at 10 or 20 mg/kg dose did not prevent the parasite reaching the brain, the results of this study demonstrated that toltrazuril sulfone is effective in preventing and treating toxoplasmosis. In a related study, it was also shown that toltrazuril sulfone at 20 mg/kg prevent recrudescent toxoplasmic encephalitis in interferon-γ knockout mice following experimental challenge (Mitchell *et al.*, 2006)

The efficacy of a single dose of toltrazuril sulfone given after challenge with *S. neurona* sporocysts for the prevention of CNS infection and clinical disease in INF-γ-knockout mice has been recently investigated (Franklin *et al.*, 2003). In this study, toltrazuril sulfone was administered once by oral gavage (either 20 or 200 mg/kg) at day 1, 3, 7, 10, or 14 post infection. All challenged mice, regardless of treatment, developed histological evidence of CNS infection even though clinical signs were prevented in some groups. The greatest treatment benefits were seen in mice given 200 mg/kg toltrazuril sulfone between days 4 and 14 post infection. The results of this study showed that protection against the experimental challenge was most effective when treatment was given 7 days after challenge and the higher dose (200 mg/kg) was more protective than the lower dose (20 mg/kg). A single dose of toltrazuril sulfone was not sufficient to eliminate the high *S. neurona* challenge used in this study, but the results of this study showed that *S. neurona* appeared to be most susceptible 7-9 days post infection (during extraneural schizogony), but not during sporozoite migration (1 to 3 days) or after invasion of the CNS (beginning at 11 days), and medications for the prevention of infection might best be administered every 1-3 days in order to be effective.

4.C. Possible mode[s] of actions of Clazurils:

4.C.1. The Apicomplexan, plastid-like chloroplast (apicoplast) organelle:

The phylum Apicomplexa (unicellular protozoa) consists of over 5,000 named species in at least 300 named genera (Levine, 1988). All members of the phylum Apicomplexa are obligate intracellular parasites. The most important members of these genera include *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, *Babesia*, *Theileria*, *Isospora*, *Perkinsus*, *Sarcocystis* and *Neospora* species which infect humans and animals causing significant

morbidity and mortality (Jeffries & Johnson, 1996).

The members of these genera are called apicomplexa due to their apical complex structure. It has been shown that organelles localized in the apical domain of parasites are involved in host-cell invasion (Dubremetz *et al.*, 1998). In this study, it was reported that exocytosis of three different secretory organelles are required for invasion of a host-cell by apicomplexan parasites; namely micronemes, rhoptries and dense granules. Apparently, micronemes are used for host-cell recognition, binding, and possibly motility; rhoptries for parasitophorous vacuole formation; and dense granules for remodeling the vacuole into a metabolically active compartment (Dubremetz *et al.*, 1998).

A number of parasitic apicomplexans contain a plastid-like chloroplast organelle variously called respectively "Hohlzylinder", "apicoplast", "double-walled", golgi-adjunct organelle", but its function and even its status in replicating cellular organelles are not known (Siddall, 1992). More interestingly, apicomplexans, in addition to their nuclear genome (chromosomes of ~ 1 to >10 Mb), possess two extrachromosomal DNAs (episomes) (Wilson & Williamson, 1997; Jeffries & Johnson, 1996). One of the episomes is a multicopy, linear DNA molecule (6 kb) known as mitochondrial DNA, and the other episome is a 35-kb circular DNA known as plastid-like DNA. Based on the molecular phylogenetic evaluation of the gene encoding elongation factor Tu (*tuf*A) in circular DNA, it was proposed that apicomplexa parasites acquired the plastid like material by secondary endosymbiosis, probably from a green alga (Kohler *et al.*, 1997).

The apicomplexan's apicoplast DNA is significantly smaller than the plastid episomes of land plants, but similar to those from non-photosynthetic organisms (Jeffries & Johnson, 1996). The plastid genomes have lost the genes required for photosynthesis, but retain the genes required for transcription and translation. On the other hand, some other molecules that are encoded by genomic DNA and localized in the apicoplast of apicomplexans have been identified in *T. gondii* and *S. muris* (Hackstein *et al.*, 1995). Based on a partial sequence of the *psbA* gene encoding chlorophyll D1 protein by PCR from *S. muris*, it was proposed that sensitivity of apicomplexans to the herbicide related toltrazuril is due to the chlorophyll D1 protein of photosystem complex II. As will be discussed later, other researchers have not been able to detect the presence of either this gene or an encoded protein that could be specifically targeted by toltrazuril and diclazuril. The same study also indicated the possibility of toltrazuril having specific antiprotozoal activity at a concentration that was effective in killing apicomplexans. This hypothesis was supported by the lack of deleterious effects of toltrazuril against trichomonads, which lack both the apicoplast and mitochondrial organelle. Further studies were recommended to determine the likely metabolic function of this organelle in apicomplexans as a possible chemotherapeutic target.

In conclusion, the sub-cellular location, expression, function, and evolution of the apicoplast and plastid-like DNAs of apicomplexans are not fully understood. Additionally, the universal presence of apicoplast and circular DNA in all apicomplexans has not been established. Even though the function of the apicoplast is not fully known, it has been pointed

out that it is unlikely that the apicoplast and its episome exist as non-functional vestigial units in apicomplexans, because in such a situation cellular resources would gradually become depleted and therefore be selected against (Feagin, 1994).

POSSIBLE MITOCHONDRIAL SITE OF ACTION OF DICLAZURIL:

Based on the literature review and the inability of other researchers to determine the presence of chlorophyll D1 protein or other specific target proteins for triazine-based agents, and also many unknown facts about apicoplasts, the exact mechanism of the antiprotozoal activities of triazine-based antiprotozoal agents remains unknown. Because of the interaction of toltrazuril with the mammalian cell's mitochondrial electron transport chain and pyrimidine *de novo* synthesis, also, the lack of efficacy of toltrazuril against trichomonads, which lack both apicoplasts and mitochondria, it is possible that toltrazuril and other clazurils interfere with the activity of apicomplexan mitochondrial electron transport chain and/or pyrimidine *de novo* synthesis.

Many studies have been conducted to indicate the importance of mitochondrial function for energy metabolism and pyrimidine *de novo* synthesis in apicomplexans. Based on the literature reviews, it is appropriate to emphasize the importance of mitochondrial function for the parasite's survival. It is especially noted that compounds interfering with apicomplexan's mitochondrial function have the ability to inhibit protozoal growth (Fry & Williams, 1984; Fry *et al.*, 1984; Hammond *et al.*, 1985; Fry & Pudney, 1992; Margaret *et al.*, 1992; Shikano *et al.*, 1998).

As mentioned earlier, one of the extra-chromosomal DNAs of apicomplexans is small (6-7 kb), linear and exists in high copy number representing the mitochondrial genome. This extra-chromosomal DNA contains genes encoding for three classical mitochondrial proteins: cytochrome *b* (*Cyp b*), subunit I of cytochrome *c* oxidase, and the less well conserved subunit III of cytochrome *c* oxidase mainly seen in *P. falciparum*. Additionally, this linear DNA encodes for fragments, six of which show homology with small subunit rRNAs and seven of which show homology with large subunit rRNAs. (Feagin, 1992).

The six enzymes that are involved in pyrimidine synthesis (uridine monophosphate, UMP) in mammalian cells have also been found in various apicomplexans. Similar to the case in mammalian cells, the fourth enzyme dihydroorotate dehydrogenase of pyrimidine *de novo* synthesis is also known to be associated with the mitochondrion's electron transport chain through electron acceptor ubiquinones (Gutteridge *et al.*, 1979). The importance of the mitochondrial function in pyrimidine *de novo* synthesis and overall energy metabolism of *B. bovis* has also been shown in a similar study (Gero *et al.*, 1983). In this study, it was also shown that the compounds that specifically interact with the protozoan mitochondrial electron transport chain have the ability to inhibit protozoan growth and pyrimidine *de novo* synthesis. There is no study that correlates mitochondrial function and pyrimidine *de novo* synthesis with *S. neurona's* growth and survival.

It has been suggested that toltrazuril and the related compound diclazuril may be selectively toxic for apicomplexans but not for mammalian cells (Hackstein *et al.*, 1995). In this study, it was shown that the apicomplexan parasite *S. muris* contains a plastid-like chloroplast organelle and a chlorophyll D1 protein, bound to photosynthetic reaction center II, for binding of therapeutic triazines. A *PsbA* gene was isolated from genomic DNA of merozoites of *S. muris* based on partial sequencing of the PCR product (20%). It was concluded that the sensitivity of apicomplexans to the herbicide toltrazuril was likely due to interaction with the D1 protein, which does not include the N terminus domain, of the photosynthetic reaction center of the parasite's organelles. On the other hand, some other researchers were unable to obtain a corresponding PCR product using malarial DNA as template and further confirmatory studies were suggested (Wilson *et al.*, 1997). Some other researchers have indicated that the presence of the *PsbA* gene could be due to contamination of the experiments with a photosynthetic organism and their attempt to determine the presence of this gene was unsuccessful. (Jeffries & Johnson, 1996). There is no study that confirms the presence of this protein and gene in apicomplexans, and one other researcher's attempts have not been successful in indicating the presence of the *PsbA* gene or chlorophyll D1 protein in other apicomplexans (Kohler *et al.*, 1997).

There are limited number of studies related to the activities of toltrazuril and diclazuril against the mitochondrial electron transport chain and pyrimidine *de novo* synthesis of mainly mammalian systems. Based on electron microscopic examination of apicomplexan parasites in the presence of diclazuril and toltrazuril, alterations in the structure of the protozoans have been reported in a few studies. It has been shown that diclazuril does not influence the growth phase, including nuclear divisions in schizonts and microgamonts, but differentiation of microgamonts and schizonts was interrupted (Verheyen *et al.*, 1988). In a similar study with toltrazuril, it was shown that the perinuclear space, mitochondria and endoplasmic reticulum are the major sites of action of this triazine, and by inhibiting nuclear division in microgamonts and schizonts, toltrazuril greatly reduces parasite production (Mehlhorn *et al.*, 1984). , Based on electron microscopy-determined alterations in the structural components of apicomplexans, both studies concluded that it is possible for triazines to be introduced into the nucleic acid synthetic system in parasites, thereby preventing nuclear growth and further differentiation. In a related study, the effects of 5 μg/ml toltrazuril sulfone on merozoites of *S. neurona* were investigated in order to determine the mode of action of this anticoccidial (Mitchell *et al.*, 2005). In this study, it was shown that toltrazuril sulfone inhibited development of merozoites of *S. neurona* and treated merozoites and maturing schizonts developed vacuoles and underwent degeneration. The ability of *S. neurona* schizonts to undergo cytokinesis was inhibited. On the other hand, this study did not determine the possible mode of action of toltrazuril sulfone, but the results of this study suggested that the systems that are inhibitory targets of toltrazuril sulfone may be different in different apicomplexans, or the results of inhibition may affect different pathways downstream of its initial site of action in different parasites (Mitchell *et al.*, 2005).

Compounds that have the ability to inhibit dihyroorotate dehydrogenase activity have been classified into three groups. Group 1 includes the analogs of quinone, L-dihydroorotate

and orotate. Group 2 includes compounds with an unknown mode of interference with dihydroorotate dehydrogenase catalysis including cinchoninic acid derivatives such as brequinar sodium, isoxazols such as leflunomide, and the herbicide toltrazuril. Group 3 includes electron transport chain inhibitors such as cyanide or azide, which would not interfere with the isolated enzyme *in vitro*, but would interrupt the flow of electrons to the final acceptor, molecular oxygen (Jockel *et al.*, 1998).

The same study Jockel *et al* has also shown inhibition of dihydroorotate oxidation in the presence of 1 μM of toltrazuril by 28% and complete inhibition in the presence of 10 μM toltrazuril in an *in vitro* rat liver study (Jockel *et al.*, 1998). More interestingly, it was shown that toltrazuril, among the six other tested compounds with known mitochondrial electron transport chain inhibition function, was the most effective one in inhibition of NADH oxidation. At a 10 μM concentration 74% inhibition was observed *in vitro*. This study also demonstrated that toltrazuril has a comparable efficacy for inhibition of NADH and dihydroorotate oxidation which contain Flavinmononucleotide (FMN) as a redox cofactor. However, 10 μM toltrazuril did not impair succinate oxidation by complex II, which contains covalently bound Flavinadeninedinucleotide (FAD). Therefore, it was concluded that the FMN and FAD structural differences could be more significant for the mode of toltrazuril action than the quinone dependence of the three enzyme systems. There are no amino acid sequence homologies of dihydroorotate dehydrogenase and the protein subunits of complex I and complex II.

A similar study was conducted in mouse liver and *Ascaris suums* mitochondrial extracts *in vitro* to determine the possible mode of action of toltrazuril. In this study, similar to one mentioned earlier, the effects of toltrazuril were studied on mammalian and nematode mitochondrial electron transport chains and pyrimidine synthesizing enzymes (Harder & Haberkorn, 1989). Interestingly, 240 μM and 24 μM toltrazuril was required to inhibit dihydroorotate-cytochrome *c* reductase activity and NADH oxidation 50%, respectively, in mouse liver *in vitro*, while 10 μM totrazuril was able to cause complete inhibition of dihyroorotate oxidation and 74% inhibition of NADH oxidation in rat liver *in vitro*. The results of these two studies indicate likely species-specific susceptibility of mammalian cellular mitochondrial electron transport chain and pyrimidine synthesizing enzymes to toltrazuril. There is no study that indicates interaction of the diclazuril or toltrazuril sulfone with the mentioned enzyme systems in both mammalian cells and apicomplexan parasites.

The third study that confirms the interaction of toltrazuril with the mammalian electron transport chain and dihydroorotate dehydrogenase enzyme activity has been shown by utilizing histochemistry and biochemical methods (Loffler *et al.*, 1996). In this study, it was also shown that the amount and activity of dihyroorotate dehydrogenase in the mitochondrial membrane is not constant and differs considerably, the highest activities of enzyme being found in tissues with known proliferative, regenerative and absorptive activities due to demand for nucleic acid precursors for cell proliferation. This study also strongly suggests that dihydroorotate dehydrogenase is a further target for the antiparasitic drug toltrazuril, in addition to its effect on Complex I of the respiratory chain.

It has been shown that when toltrazuril was combined with either trimethoprim or pyrimethamine, there was a clear synergism of antiprotozoal activity compared to each compound used alone in *E. falciformis*-infected mice *in vivo* (Harder & Haberkorn, 1989). Similar results were obtained when *E. tenella*-infected chicken kidney cell culture was used *in vitro* (Harder & Haberkorn, 1989). The synergistic antiprotozoal activity of the combination of diclazuril with pyrimethamine has also been reported against *T. gondii*-infected mice *in vivo* (Lindsay *et al.*, 1995b). In this study, all *T. gondii*-infected mice subject to this combination therapy (0.5-1.5 mg/kg diclazuril with 12.5 mg/kg pyrimethamine) survived the 56-day observation period. It is believed that compounds that exert their specific actions at different sequential points of a particular metabolic pathway are more likely to have the ability to potentiate each other's actions. It is known that pyrimethamine and trimethoprim inhibit activity of the dihydrofolate reductase enzyme that is involved in pyrimidine *de novo* synthesis in apicomplexans. Therefore, synergistic antiprotozoal activities of toltrazuril and diclazuril with these compounds may be due to the ability of triazines also to interfere with the pyrimidine *de novo* synthesis in parasites.

4.C.2. APICOMPLEXANS VERSUS MAMMALIAN CELLS:

Major differences between parasitic apicomplexans and higher eukaryotes exist in their pyrimidine *de novo* synthetic pathway. The first three enzymes in pyrimidine *de novo* synthesis are carried on separate proteins in the parasites, whereas they exist as a multifunctional protein in most eukaryotes (Krungkrai, 1995). The fourth enzyme dihydroorotate dehydrogenase is also found to be physicochemically and kinetically different from that reported in mammalian cells, and also the last two enzymes of the pathway are encoded by two separate genes in some apicomplexans, while in mammalian cells they exist as a bifunctional protein. But whether or not these organizational differences can result in significant functional differences is still not known. Additionally, while these enzymes are located in the inner mitochondrial membrane of some apicomplexans and higher eukaryotic cells, some cytosolic forms of these enzymes have been reported in *Plasmodium* species.

Targets unique to the organism or possessing altered properties from that of the host cells are good candidates for the development of parasite-specific inhibitors. It is worth mentioning that trimethoprim and pyrimethamine inhibit the activities of dihydrofolate reductase and thymidilate synthase in the parasite, while they have very little effects on the corresponding mammalian enzymes.

It is known that separate genes in mammalian cells encode these two enzymes, while they are fused and a bifunctional enzyme is produced in apicomplexans (Kisthardt & Lindsay, 1997). Because of these organizational and structural differences between mammalian and apicomplexans enzymes, pyrimethamine and trimethoprim have more activity against apicomplexans enzymes. Similarly, the anti-malarial agent cycloguanil has a several hundred-fold higher affinity for the malarial dihydrofolate reductase than for the mouse enzyme. . These structural and kinetic differences for pyrimidine *de novo* synthesizing enzymes and encoding

genes, between mammalian and apicomplexans, may account for selective activities of triazine-based antiprotozoal agents with selective toxicity against parasites. There is no study that compares the sensitivity of the mammalian enzyme systems with those of apicomplexans for toltrazuril, diclazuril or toltrazuril sulfone.

Examination of the supernatant of isolated *P. falciparum* led to the finding that enzyme activities involved in UMP *de novo* synthesis are much higher than in the host cells (erythrocytes) (Gero & O'Sullivan, 1990). The rapidly dividing cell's requirement for pyrimidine *de novo* synthesis is higher than host cells (in this situation erythrocytes). It was also reported that most of the host cells, except red blood cells, do have the ability to salvage preformed pyrimidine bases, nucleosides, or synthesize their own, while some apicomplexans, like *P. falciparum*, lack enzymes responsible for salvaging preformed pyrimidines, therefore their survival exclusively depends on the pyrimidine *de novo* synthesis. This could be another point for selective activity and toxicity of triazine-based agents against apicomplexans.

As mentioned earlier, mitochondrial DNAs of apicomplexans encode three mitochondrial proteins. One of them, cytochrome *b*, is a component of the bc_1 complex of the electron transport chain, and the site of electron acceptor from ubiquinone. (Feagin, 1994). The amino acid sequence difference between the plasmodium and mammalian cytochrome *b* protein was suggested as the reason for greater affinity of some hydroxynaphthoquinones and 8-aminoquinolines for the parasite bc_1 complex. Additionally, amino acid sequences for subunit I and subunit III of cytochrome *c* oxidase were reported to be similar to those in humans, 73% and 31%, respectively.

When all these facts are taken into consideration, it is possible that triazine agents selectively decrease and/or inhibit the synthesis of nucleotide triphosphates (NTP) and deoxynucleotide triphosphates (dNTP) as a consequence of interfering with the pyrimidine *de novo* synthesis pathway, and therefore lead to miscoded genes and/or arrest of DNA and RNA synthesis in parasites. Clearly, further studies are required to determine the effects of toltrazuril and the related compound diclazuril in apicomplexan mitochondrial electron transport chain activity and pyrimidine *de novo* synthesis. Finally, even though, the specific basis for the antiprotozoal activity of triazines is not known, the data presented in the literature very strongly indicates that these compounds are highly selective against apicomplexan parasites with very little toxicity in/for mammalian systems.

4.D. Pharmacokinetics, Clinical Safety and Efficacy of Clazurils in Horses:

In earlier studies we identified diclazuril as a potentially important therapeutic agent for use in the treatment of EPM (Granstrom *et al.,* 1997; Bentz *et al.*, 1998); as such we needed to perform preliminary bioavailability and pharmacokinetic experiments with this agent in the horse. Following single oral administrations of diclazuril in DMSO, diclazuril as a sodium salt, (Dirikolu *et al.*, 2006), toltrazuril as Baycox® (Tobin *et al.*, 1997), toltrazuril sulfone as a sodium salt (Dirikolu 2011, unpublished data) and toltrazuril sulfone in DMSO (Dirikolu *et al.*, 2009), analysis of plasma samples indicated relatively low variability in both peak plasma

concentrations and plasma half-lives of these agents in the horse. On the other hand, when diclazuril as Clinacox®, an avian feed pre-mix, was administered orally to four horses, peak plasma concentrations and plasma half-life showed considerable inter-animal variability (Dirikolu *et al.*, 1999). Since we had detailed information related to clinical efficacy of diclazuril as Clinacox®, it was possible to make correlations between the kinetic parameters and clinical efficacy of this compound in the treatment of EPM. The mean plasma concentrations of diclazuril in the four horses tested in these experiments showed considerable horse to horse variability, with the range of the peak concentrations falling between 750 and 1600 ng/ml at 24 hours post-dosing (Dirikolu *et al.*, 1999). Similarly, the apparent plasma half-lives of diclazuril in each horse varied from a low of 26 hours to a high of 59 hours with the average half-life being approximately 43 hours (Dirikolu *et al.*, 1999). Our Clinacox® data suggested that repeated oral administration of diclazuril would give rise to useful steady state plasma concentrations of this drug, although significant variability in the final steady state concentrations between individual horses was to be expected. More importantly, however, these well-maintained steady state plasma concentrations of diclazuril should enable diclazuril to enter the central nervous system of horses and achieve steady state concentrations in the cerebrospinal fluid.

It should be borne in mind that these experiments suggest that the oral bioavailability of diclazuril administered as Clinacox® may vary between individual horses in a clinically significant manner. For example, in our small sample of 4 horses there was a two- fold difference between the peak plasma concentrations of diclazuril as Clinacox® observed in the high (1600 ng/ml) and the low (750 ng/ml) horses (Dirikolu *et al.*, 1999). These differences presumably translate into equivalent differences in steady state concentrations of diclazuril attained in plasma and ultimately in the CSF of treated animals. For example in our clinical efficacy study, when the dosage was adjusted from 5 mg/kg to 5.5 mg/kg and the treatment interval was extended from 21 days to 28 days to allow for longer duration of detectable CSF level (6 horses in our clinical efficacy trial), the rate of post-treatment relapse rate was reduced indicating the importance of oral absorption of diclazuril in horses (Bentz *et al.*, 2000).

These apparent differences in the bioavailability of Clinacox® may represent one possible source of apparent therapeutic failure with diclazuril or, more likely, a possible source of short term "relapses" after completion of a course of therapy with this agent. This is because in any large series of experimental animals there will be some animals that will have difficulty in absorbing sufficient diclazuril from orally administered Clinacox® to yield fully effective plasma and CSF therapeutic concentrations of this agent. Three possible solutions to this problem present themselves. The most practical one is the development of a highly bioavailable oral preparation of diclazuril which would reliably yield effective plasma and CSF concentrations of this agent in all horses treated. A secondary approach is to monitor plasma concentrations of diclazuril during therapy and adjust the oral dose to compensate for any bioavailability deficits associated with individual animals. A third approach is to develop a highly bioavailable oral preparation of a diclazuril-related compound, which will routinely yield effective plasma and CSF concentrations of this agent in all horses treated. Additionally, an improved formulation would allow the clinician to administer a loading dose without

worrying about the relative amount of the drug in acute cases of EPM. On the other hand, either toltrazuril as Baycox® and especially diclazuril as Clinacox® are not suitable to be loading dose administrations to rapidly attain effective therapeutic concentrations of these agents. Since DMSO was relatively safe to use and the parenteral administration of DMSO enhances the ability of high molecular-weight substances to be absorbed, we chose DMSO as the solvent to keep our triazine-based agents in solution. Additionally, we also evaluated sodium salt formulations of triazine-agents in order to enhance oral bioavailability of these compounds.

In our study we demonstrated that DMSO increases the solubility and absorption characteristics of triazine-based agents in the horse following oral administration. Both diclazuril and toltrazuril sulfone had relatively high solubility in DMSO, 100 mg/ml and 150 mg/ml, respectively. The mean peak plasma concentrations of diclazuril at 24 hours following oral administration of 2.2 mg/kg in DMSO was approximately one and half times higher than that of following oral administration as Clinacox® (5 mg/kg) (Dirikolu *et al.*, 2006). The relative bioavailability of diclazuril as Clinacox® compared to diclazuril in DMSO was 20%, indicating approximately five-fold reduction in bioavailability of diclazuril when administered n as Clinacox®. On the other hand, analysis of plasma samples from horses dosed with IM injection of diclazuril in DMSO showed lower plasma levels of diclazuril relative to oral administration. In this study, it was also found that sodium salt formulations of triazine-based agents are well absorbed following oral-mucosal administration (Dirikolu *et al.*, 2006). Analysis of the plasma samples showed rapid absorption of diclazuril following oral-mucosal applications of diclazuril sodium salt (2.2 mg/kg) to four horses with a mean observed peak plasma concentration of 3,930 ± 154 (SEM) ng/ml of diclazuril at 24 h after administration. Additionally, the relative bioavailability of diclazuril as Clinacox® ranged from 5.24% to 13.65% relative to diclazuril sodium salt, with mean bioavailability of about 9.3 +/- 2% (SEM). The relative bioavailability of diclazuril in DMSO ranged from 30% to 76% relative to diclazuril sodium salt with the mean bioavailability of about 49 +/- 10% (SEM) (Dirikolu *et al.*, 2006).

To further investigate the effect of DMSO on the absorption characteristics of triazine-based agents, we compared the results of oral administration of toltrazuril sulfone in water and DMSO (Dirikolu *et al.*, 2009). It was found that the mean peak plasma concentration of toltrazuril sulfone at 24 hours following oral administration in DMSO was approximately four times higher than following oral administration in water. The relative bioavailability of toltrazuril sulfone in water compared to in DMSO was 33% indicating an approximately three-fold reduction in the bioavailability of toltrazuril sulfone following oral administration in water versus in DMSO. The mean bioavailability of toltrazuril sulfone in DMSO was 71% following oral administration in DMSO (Dirikolu *et al.*, 2009). Daily administration of toltrazuril sulfone orally (2.2 mg/kg in DMSO) to two horses yielded steady state concentrations of toltrazuril sulfone in the cerebrospinal fluid samples between 125-220 ng/ml. Daily administration of toltrazuril sulfone IV (2.2 mg/kg in DMSO) to two horses yielded steady state concentrations of toltrazuril sulfone in the cerebrospinal fluid samples between 150-230 ng/ml. Additionally, in our 28-day treatment schedule, none of the horses showed any clinical signs of toxicity

related to administration of toltrazuril sulfone in DMSO (Dirikolu *et al.*, 2009).

There are a limited number of publications related to absorption, distribution, and metabolism characteristics of triazine-based antiprotozoal agents in the horse. Based on our initial pharmacokinetic research, other researchers have confirmed the therapeutically sufficient absorption characteristics of this group of agents following oral administration (Furr & Kennedy, 2000). In this study, following oral administration of toltrazuril as Baycox® at 10 mg/kg, a rapid initial absorption was found, with mean peak serum concentrations of 11.17 μg/ml at 18 hours. Similar to our findings, these researchers also confirmed that toltrazuril has a relatively long plasma half-life following oral administration in the horse. This study also therefore confirmed that triazine-based antiprotozoal agents are well absorbed and, due to their long plasma half-lives, daily oral dosing readily yields therapeutically useful and consistent steady state plasma and CSF concentrations. Mean CSF concentrations of toltrazuril after 10 days of treatment were 0.146 μg/ml, 0.190 μg/ml and 0.386 μg/ml from nine horses dosed orally with 2.5 mg/kg, 5 mg/kg and 7.5 mg/kg, respectively (three horses for each treatment schedule) (Furr & Kennedy, 2000). Furr and Kennedy (2000) also reported that toltrazuril sulfone and toltrazuril sulfoxide were the predominant metabolites following oral administration of toltrazuril as Baycox®. Mean steady state concentrations of toltrazuril sulfone in CSF after 10 days of treatment were 0.09 μg/ml, 0.157 μg/ml, and 0.223 μg/ml following daily oral doses of toltrazuril at 2.5 mg/kg, 5 mg/kg, and 7.5 mg/kg, respectively. There were no signs of toxicity of toltrazuril in this study following two months or longer oral administration of toltrazuril (Furr & Kennedy, 2000). In a related study, toltrazuril sulfone, using the commercially available 15% oral paste, was administered orally to 10 horses at 5 mg/kg, once a day for 28 days (Furr & Kennedy, 2001). Toltrazuril sulfone was relatively well absorbed following 7 days of dosing, and the serum concentration was 4.33 +/- 1.10 μg/ml, and the mean CSF concentration was 162 +/- 5 ng/ml. This study also confirmed that triazine agents have relatively long elimination half-life in the horse with the elimination half-life of toltrazuril sulfone (using Day 28 and 42 results) in this study being 4.3 +/- 0.6 days (Furr & Kennedy, 2001).

Consequently, many veterinary drug product labels now contain pharmacokinetic information to better support practitioners in exercising their therapeutic options (Martinez *et al.*, 1995). Steady state conditions can be considered as having been achieved when concentrations reach 94% of their steady state value within approximately four half-lives (Benet & Zia-Amirhosseini, 1995). Based on the mean-plasma half-lives of diclazuril, toltrazuril and toltrazuril sulfone following oral administrations, the time required to achieve steady state plasma concentrations are approximately 7 days for diclazuril as Clinacox®, 8 days for toltrazuril as Baycox®, approximately 2 weeks for toltrazuril sulfone and diclazuril in DMSO, and diclazuril sodium salt, respectively. Based on the range of the plasma half-lives of triazine-based antiprotozoal agents following oral administrations, the time range required to obtain steady state plasma concentrations following daily oral administration will be 4-10 days for both diclazuril as Clinacox® and toltrazuril as Baycox®, 11-18 days for toltrazuril sulfone in DMSO, 12-13 days for diclazuril sodium salt, and 9-26 days for diclazuril in DMSO. *In vitro* studies conducted on the sensitivity of *S. neurona* for triazine-based agents are usually

performed approximately 10 to 13 days following incubations. Since there will be variation in times required to achieve steady state plasma and CSF concentrations among treated horses, it may be necessary to extend the treatment duration for some individuals with a relatively long plasma half-live in order to maintain MIC of the agent in the CNS for a therapeutically sufficient period of time. It is also important to consider that there might be an alteration in the behavior of the drug when it is tested in animals *(in vivo)* and in culture assay (*in vitro*). Certain factors including serum drug concentration, host cell type selected for examination, passage number, specific protozoal strain, duration of observation following incubation with drug, and testing method for drug efficacy along with complications introduced by the host cell immune response, can alter the efficacy of the drug in both *in vitro* and *in vivo* assays (MacKay *et al.*, 2000). Since the length of time the drug concentration exceeds the MIC in the CNS is considered one of the most important pharmacokinetic variable for a clinical efficacy study, it may well be advisable to extend the treatment period for agents or individuals in which the compound in question has a long plasma half-life. It remains unknown what effect extension of the treatment period has on clinical efficacy and especially in clinical toxicity of the triazine-based antiprotozoal agents in question. Clinicians may wish to consider administration of loading doses of these agents, especially in acute cases of the disease. For example, in our clinical efficacy study following relapse of 2 horses, the dose of diclazuril as Clinacox® was adjusted to 5.5 mg/kg and the treatment was extended from 21 days to 28 days. Six horses received the adjusted dosage for 28 days. Four horses showed significant improvement following this treatment schedule. On the other hand, the positive response to this treatment schedule may be due to extension of treatment schedule, or the increased dose, or to both. Therefore, the effect of extension of the treatment period effect on the clinical efficacy of these agents remains unknown.

Pharmacokinetic analysis following single oral administration provided information regarding the disposition and half-life of triazine-based agents in the plasma and CSF of the horse. This information, together with the *in vitro* sensitivity of *S. neurona* to triazine-based agents, was used to design and test dosage schedules required to maintain effective steady state plasma and CSF concentrations of these compounds in horses. The initial doses for diclazuril as Clinacox® (5mg/kg) and toltrazuril as Baycox® (10 mg/kg) were derived from review of literature suggesting that coccidiocidal dosages of these compounds ranged from 1 to 10 mg/kg of body weight (Lindsay & Blagburn, 1994; Hackstein *et al.*, 1995; Lindsay *et al.*, 1995b).

In a clinical efficacy study of diclazuril as Clinacox® for EPM, forty-four horses were included in the investigation. Neurological and diagnostic evaluations were obtained from board-certified internists (Bentz *et al.*, 2000). Thirty-seven of the 44 (84%) cases had been previously diagnosed and treated with standard treatment pyrimethamine/sulfadiazine therapy and seven had not. Diclazuril as Clinacox® was administered to horses at a dose of 5 mg/kg of diclazuril suspended in 6-8 L of water by nasogastric intubation or adding the premix to the daily feed for 21 days. Following relapse of 2 horses, the dose was adjusted to 5.5 mg/kg and the treatment was extended to 28 days. Six of the 44 horses received the adjusted dosage of 5.5 mg/kg for 28 days. Twenty-eight of the 40 horses (70%) that completed treatment were improved. Four of the six horses receiving 5.5 mg/kg diclazuril for 28 days improved. Twelve

of the 40 (30%) horses failed to improve with treatment. Of seven horses that had not been previously treated for EPM prior to investigation, three improved (42.9%) and four failed to improve (57.1%). The cerebrospinal fluid immunoblot analysis was performed on 8 of the 40 treated horses at 6 to 12 months after initial treatment with diclazuril. Two horses were CSF negative and 6 horses remained positive. No other repeat spinal fluid western blots have been reported.

Adverse effects associated with treatment reported on the 6 months follow-up questionnaire included; worsening of clinical signs (7/40, 17.5%), colic (1/40, 2.5%), mild elevation of liver enzymes (AST, GGT) (2/40, 5%). Since blood work was not performed on a majority of the cases included in the investigation, it is difficult to determine true occurrence of increases in serum liver enzymes. Of the seven horses that exhibited worsening of clinical signs with treatment, 2 were initially grade 5, 3 were initially grade 4, 1 was grade 3 to 4, and 1 was grade 2 (Bentz *et al.*, 2000).

Triazine-based antiprotozoal agents are used for the treatment and prophylaxis of a wide variety of apicomplexans diseases. The exact mechanism of antiprotozoal activity of these agents is not known. Limited work in other species has indicated minimal toxic effect of diclazuril and toltrazuril at high doses for several consecutive days. Acute toxicity trials for diclazuril on rats, mice, dogs and hens failed to produce drug-induced mortality (Janssen Pharmaceutica Safety Services, 1995). No mutagenic, teratogenic or carcinogenic affects for diclazuril have been demonstrated. The findings of our clinical trial in the horse support our prediction of low toxicity in this species. Adverse reactions reported in our investigation were few, and could not be specifically linked to the compound, and the small number of cases of worsening of clinical signs with treatment appeared to be largely in the more severely affected horses.

At 6 months after completion of treatment, relapse was reported in 5% (2/40) of the horses that completed treatment (Bentz *et al.*, 2000). Horses that exhibited relapse were suspected to have been under-dosed. Re-treatment of both horses was performed with the adjusted dosage of 5.5 mg/kg for 28 days. This resulted in positive sustained improvement (at least 1 neurologic grade) in both animals. None of the 6 horses that initially received 5.5 mg/kg for 28 days had relapsed 6 months after treatment. Although the numbers are not directly comparable, the over-all relapse rate shows greater efficacy than a retrospective study in which EPM was treated by standard therapy (pyrimethamine/sulfonamides). In clinical investigation of standard treatment, a 37% relapse rate was reported between 2 weeks and 6 months after discontinuation of treatment in a population of horses treated for various lengths of time (Fenger *et al.*, 1997b).

Toltrazuril sulfone recently has been evaluated for the treatment of EPM in a study sponsored by Bayer Animal Health (Pittsburgh, PA) (Furr *et al.*, 2001). Approximately 100 horses that had not been previously treated for EPM were treated for 28 days with a 15% paste formulation of toltrazuril sulfone either at 5 or 10 mg/kg. Overall, 62% of horses improved with no signs of toxicity. The Western blot for CSF became negative in 10% of the horses.

CSF concentration of toltrazuril sulfone was found to be 150-180 ng/ml from days 7-28, falling to 20 ng/ml 7 days after treatment following oral administration of toltrazuril sulfone at 5 mg/kg/day (Furr *et al.*, 2001).

The clinical efficacy studies with diclazuril and toltrazuril sulfone have shown that the 28-day course of therapy is highly effective in the treatment of this disease (60-70% of horses improved clinically). At 6 months after completion of treatment with diclazuril, relapse was reported in 5% of the horses. The rate of adverse reactions to these agents is also low, consistent with their highly selective toxicity for apicomplexans. Additionally, other work suggests that these agents can be combined with current therapy to further increase the efficacy of therapy (Harder & Haberkorn, 1989, Lindsay *et al.*, 1995b).

In a recent study, it has been shown that prophylactic administration of toltrazuril sulfone reduces clinical signs and delays seroconversion in horses experimentally challenged with *S. neurona* (Furr *et al.*, 2006). Treatment consisted of either 2.5 mg/kg or 5 mg/kg toltrazuril sulfone administered daily beginning 7 days before infection and continuing for 28 days postinfection. In this study, it was shown that all the challenged horses without treatment developed neurological signs, on the other hand, only 71% and 40% of horses in 2.5 and 5 mg/kg toltrazuril sulfone treated groups, respectively, developed neurological abnormalities. The results of this study demonstrated that pre-and continuous administration of horses with toltrazuril sulfone minimizes, but does not eliminate, infection, subsequent seroconversion and clinical signs of EPM in horses. Further studies are required in order to determine effective duration of treatment for the prophylaxis of EPM.

In a related study, the effect of intermittent oral administration of toltrazuril sulfone on experimental *S. neurona* infection of horses has been investigated (Mackay *et al.*, 2008). In this study, horses (5 horses/group) each received 612,500 *S. neurona* sporocysts via nasogastric tube, and were not treated or were treated with toltrazuril sulfone (20 mg/kg) orally every 7 days (beginning on day 5 post infection) or every 14 days (beginning on day 12 post infection). The results of this study showed that administration of toltrazuril sulfone every 7 days, but not every 14 days, significantly decreased the antibody response against *S. neurona* 17-kd antigen in CSF in horses experimentally inoculated with *S. neurona* sporocysts. In this study, none of the challenged horses, both treated and not treated, developed reliable clinical or histological evidence of CNS disease.

There are not many studies reporting the safety of triazine-based drugs in equine models. In one of the safety studies, toltrazuril sulfone, using commercially available 15% oral paste was administered to 24 horses at 0, 10, or 30 mg/kg body weight for either 28 or 56 days, representing zero, two, and six times the proposed dosage rate and one and two times the recommended duration of treatment, respectively (Kennedy *et al.*, 2001). Soft stools were observed in four of 16 treated horses and three of eight control animals. Serum blood urea nitrogen levels increased and serum sodium levels decreased in horses receiving 10 mg/kg; however, these changes were not associated with number of days on treatment and no value for either variable was outside the normal reference range. No signs of colic were observed in any

treated animal during the study. At necropsy, uterine edema was noted in three of the four mares treated with toltrazuril sulfone at 30 mg/kg. No other treatment-related postmortem or histological abnormalities were identified in any of the horses. The findings of this study suggested that toltrazuril sulfone has minimal toxic potential when dosed at levels up to six times the recommended clinical dosage for as long as 56 days.

5. Summary and Conclusions

EPM is an infectious neurological disease of American horses that affects the central nervous system (CNS) and is caused by the parasitic apicomplexan *S. neurona*. Infection with this disease can interfere with horse's ability to race, work and perform in equestrian events. . Ante-mortem diagnosis of EPM is challenging and requires Western blot analysis of serum and CSF together with rigorous clinical examination. Although there are number of commercially available tests to detect antibodies to *S. neurona*, all have similar shortcomings. Neurologic conditions other than EPM must be considered in the diagnostic evaluation of any horse exhibiting compatible neurologic deficits. Therefore, careful evaluation and interpretation of all the information obtained from the diagnostic process is very important for diagnosis. In the case of relapse and lack of response to treatment, the diagnosis of EPM should be reevaluated.

The epidemiology and economic significance of *S. neurona* infection in the United States is substantial. In endemic areas in the US, over 40% of horses (45-60%) are seropositive (Granstrom *et al.*, 1997, Bentz *et al.*, 1997, Blythe *et al.*, 1997, Saville *et al.*, 1997, Rossano *et al.*, 2001), even though the disease is considered to clinically affect less than 1% of horses. On the other hand, it is believed that economic loss due to EPM in the performance horse breeding is likely more substantial than indicated by reported EPM cases, because only clinically observable cases of EPM are reported, excluding subclinical cases. Currently, the reason why only a small percentage of infected horses develop clinical disease is unknown, and there is very little information about the nature of protective immunity against S. *neurona*.

Preventative treatment methods for EPM are currently not well defined. Even though it has been shown that triazine agents can be used prophylactically for the prevention of EPM in experimentally challenged animal models, duration of treatment and the dose required for the effective prophylaxis of EPM in naturally exposed horses have not yet been identified. Treatment of this disease is difficult since some horses may not respond; some may show lack of response and some may relapse after initial treatment success. None of the approved treatments for EPM are obviously superior with all having about 60-70% treatment success in clinical trials, although there may be significant differences in relapse rates. Pharmacokinetic studies of salt formulations of triazine agents and formulations prepared in DMSO showed clinically significant improvement in bioavailability of these agents and also point to possible use as a feed additive in the equine model. On the other hand, further studies are required in order to determine the clinical efficacy and safety of these new triazine formulations for the treatment/prophylaxis of EPM.

Numerous adjunctive therapies for EPM are also utilized. Anti-inflammatory therapy can help reduce inflammatory responses to the protozoan and may be useful in "treatment crisis" (transient worsening of clinical signs early in treatment) reported in some severe cases receiving an anti-protozoal medication. Use of corticosteroids in EPM cases is controversial. . Immune stimulants have also been recommended and include products such as Propionibacterium acnes administration, mycobacterial cell wall extracts, oral levamisole, and alpha-interferon.

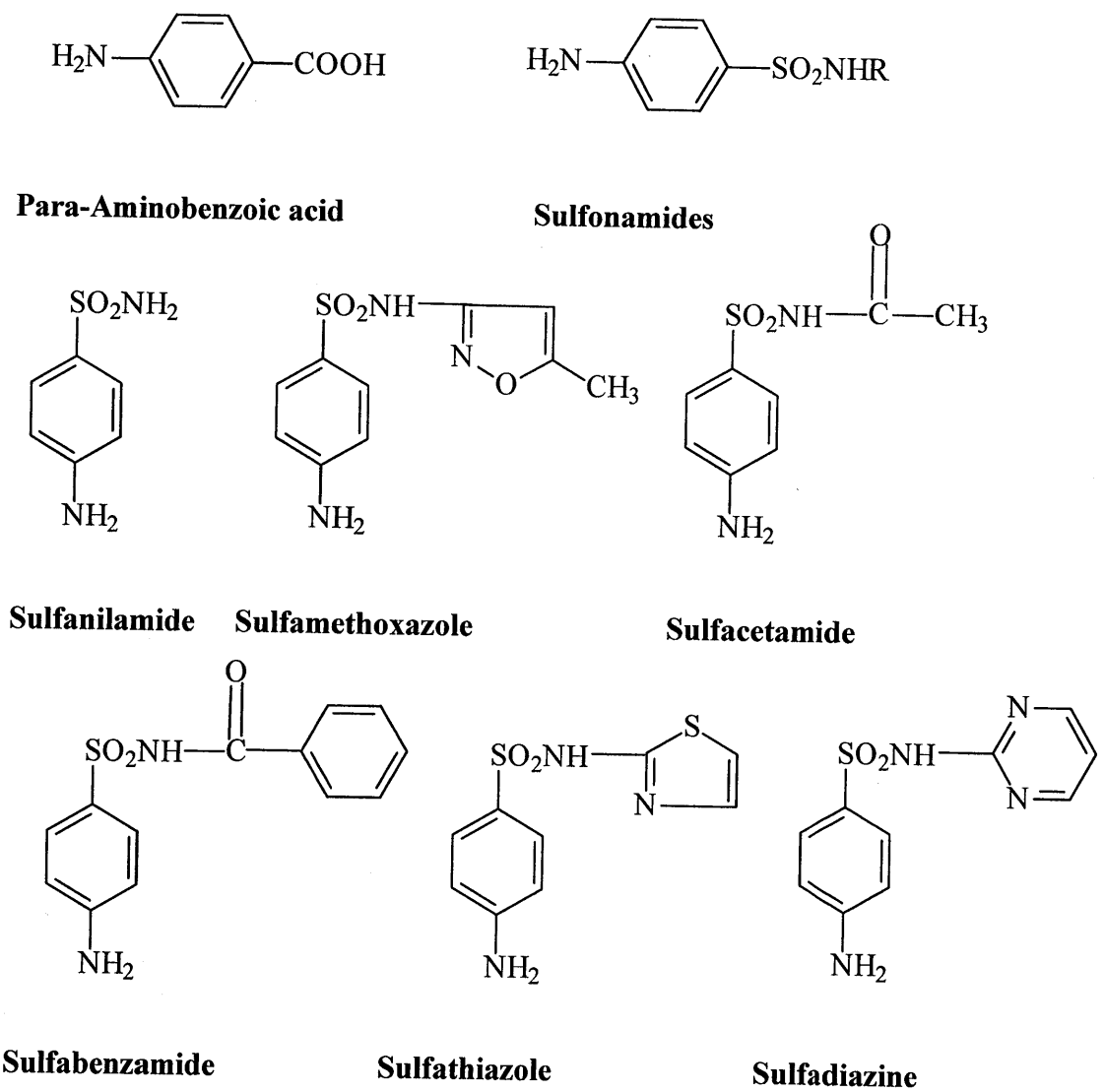
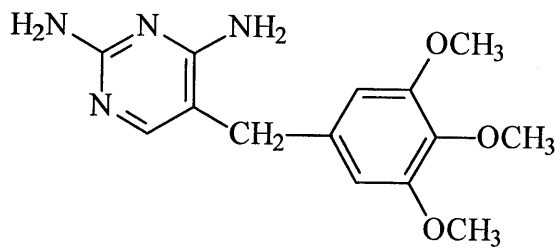
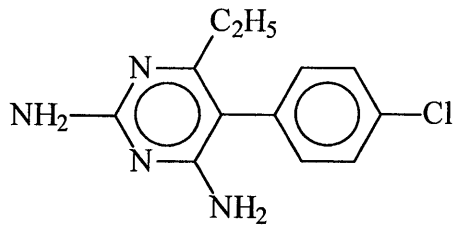


Figure 1. Structures of para-aminobenzoic acid and commonly used sulfonamides.



Trimethoprim



Pyrimethamine

Figure 2. Structures of pyrimethamine ($C_{12}H_{13}ClN_4$) and trimethoprim ($C_{14}H_{18}N_4O_3$).

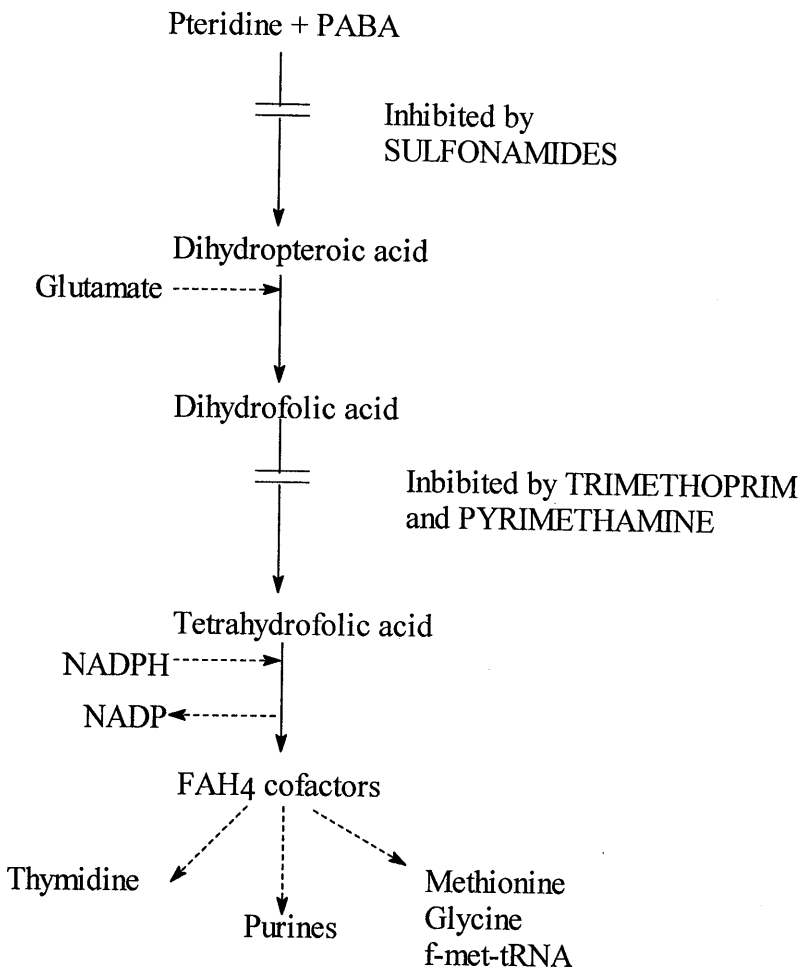


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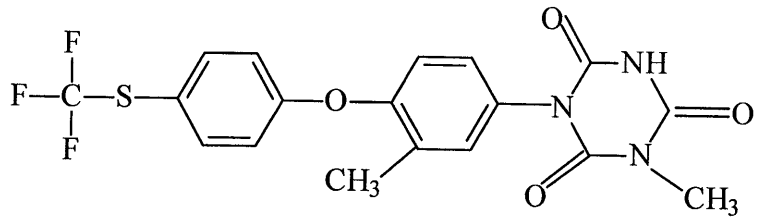
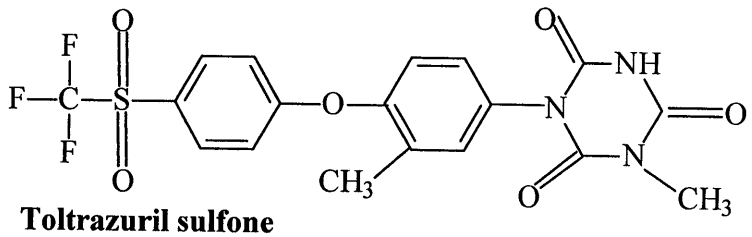
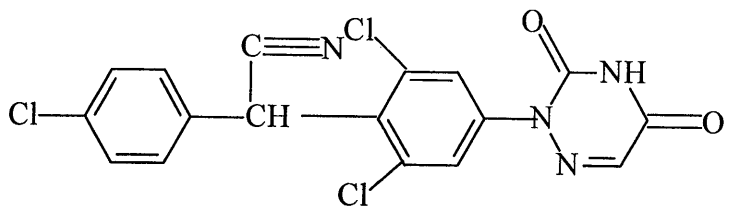
Figure 3. Mechanism of the anti-bacterial and antiprotozoal sequential inhibition activities of trimethoprim and sulfonamides. The sulfonamindes inhibit dihydropteroate synthesase in step number 1 of the sequential inhibition mechanism and trimethoprim/pyrimethamine inhibit dihydrofolate reductase in step number 2 in the sequential inhibition process.



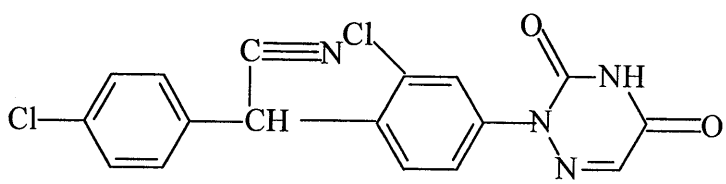
Toltrazuril



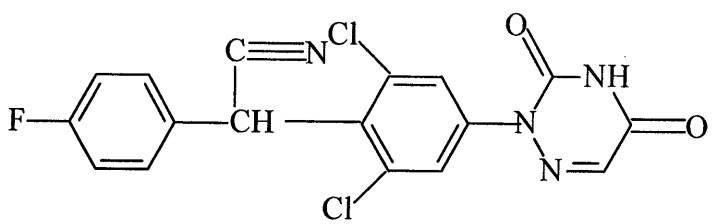
Toltrazuril sulfone



Diclazuril



Clazuril



Letrazuril

Figure 4. Structures of triazine-based antiprotozoal agents.

[1]Phizer, Inc., New York City, NY, USA
[2]Bayer Corporation, Agriculture Division, Shawnee Mission, KS, USA
[3]Animal Health, Inc., St. Joseph, MO, USA
[4] Intervet/Schering-Plough Animal Health, Summit, NJ, USA

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