IDENTIFICATION OF DIFFERING STRAINS OF SARCOCYSTIS NEURONA MEROZOITES

THESIS

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By

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ABSTRACT

Sarcocystis neurona is considered the major etiologic agent of equine protozoal myeloencephalitis (EPM), a neurological disease in horses. Raccoons (Procyon lotor) act as an intermediate host in the life cycle of S. neurona. The focus of this study was to determine if sarcocysts would develop in raccoons experimentally inoculated with different host-derived strains of in vitro cultivated S. neurona merozoites. Four raccoons were experimentally inoculated with merozoites from the isolates Sn-OT-1 (sea otter derived), Sn-37-R (raccoon derived), Sn-UCD 1 (equine derived) and Sn-Mucat2 (cat derived). Two raccoons were orally inoculated with sporocysts to act as positive controls. Raccoon tissues were then fed to laboratory raised opossums (Didelphis virginiana), the definitive host of S. neurona. Gastrointestinal scraping revealed sporocysts in two of the opossums who received muscle from the raccoons that were inoculated with the raccoon-derived or the sea otter derived isolates. These results suggest that raccoons can form tissue cysts from infection with in vitro derived S. neurona merozoites. In contrast, the equine and cat-derived isolates did not produce microscopically or biologically detected sarcocysts. All raccoons seroconverted to S. neurona as detected via immunoblot analysis. Further western blot analysis revealed antigenic differences when tested with the raccoon sera. Immunohistochemical tests also indicated antigenic differences between the merozoite and sarcocyst stages. These overall results demonstrated antigenic and biological differences between isolates and life-cycle stages. The successful infections achieved in this study indicates that the life-
cycle can be manipulated in the laboratory without affecting subsequent stage
development, thereby allowing further purification of strains and artificial maintenance of
the life cycle.
DEDICATION

This thesis is dedicated to all those who have helped me along the way, my family, friends, and teachers. For everyone who helped make this finally come together thank you for all the guidance, understanding, inspiration, and patience.
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CHAPTER 1: INTRODUCTION

*Sarcocystis neurona* is the causative agent for equine protozoal myeloencephalitis (EPM), which is a progressively debilitating neurologic disease that affects horses and ponies in the Americas. The large distribution of the disease is due in part to the fact that it utilizes a large range of intermediate hosts during the life cycle. The life cycle for *S. neurona* utilizes an obligate intracellular protozoan parasite that uses a two host cycle. The definitive host, the opossum, *Didelphis virginiana* in North America and *Didelphis albiventra* in South America, was first identified by Dubey in 2000 (Dubey et al., 2000d). The intermediate hosts that have been identified at this point include the nine-banded armadillo, raccoon, domestic cat, striped skunk and sea otter. The horse is considered an aberrant or dead end host since the sarcocysts do not form in the horse.

The opossum is an opportunistic feeder and will eat most anything that is available. Because of these feeding habits it serves as an ideal candidate for multiple parasite life cycles including serving as the intermediate host of *S. greineri* and the definitive host for several *Sarcocystis* species including *S. neurona, S. falcataula, S. speeri,* and *S. lindsayi.* It not only acts as a host but also a vector. It has a fairly large area that it will cover and therefore often comes into close proximity of farms and suburbs. While the opossum does not show any clinical signs associated with infection of *Sarcocystis,* the intermediate hosts can show neurologic signs associated with increased parasite burdens (Stanek et al. 2002, Dubey et al 1994, Dubey et al 1996).
Due to the variety of intermediate hosts involved in this life cycle and the geographic
distribution of the opossum, the disease is widely spread throughout the Americas.

Due to the large distribution of hosts it is important to understand how the
different species involved are able to continue the perpetuation of this parasite. The
purpose of this study was to better identify how varying strains obtained from the
different hosts may affect the ability for the parasite to encyst in a particular intermediate
host, such as the raccoon. By understanding the different strains of parasite, and if these
differing strains are interconnected, we will be able to better devise new methods for the
treatment and prevention of EPM.
CHAPTER 2: LITERATURE REVIEW

2.1. THE HISTORY OF EPM

Equine Protozoal Myeloencephalitis (EPM) is a severe neurologic disease that affects horses throughout North, Central and South America (Granstrom et al., 1992). The origins of EPM can be traced back to a disease called segmental myelitis syndrome that was first recognized in 1964 by Rooney et al in Kentucky (Rooney et al., 1970). The syndrome was renamed “focal encephalitis-myelitis” due to the involvement of the brain (MacKay et al., 2000). Original case reports, including 44 cases presented at the annual American Association of Equine Practitioners convention in 1968 and 52 reported cases in 1970, revealed a vast range of horses affected by the disease (Rooney et al., 1970). The horses affected ranged from 1-24 years of age with the highest incidence of the disease associated with Standardbreds. The disease also seemed to be seasonal with summer months having the highest occurrence of disease. In 1974 Mayhew and colleagues identified characteristic protozoan lesions in association with this disease; therefore, it was renamed Equine Protozoal Myeloencephalitis (EPM) (Cusick et al., 1974; Mayhew et al., 1976).

Preliminary work regarding the identification of the parasite focused on the elimination of potential Sarcocystis species. The majority of these efforts were focused on a trial and error method during which horses were orally inoculated with different presumed Sarcocystis species (Fayer and Dubey, 1987). Multiple common Sarcocystis
spp, including *S. fayeri*, *S. cruzi*, and *S. tenella* were ruled out after oral inoculation with these parasites failed to reproduce clinical signs associated with protozoal infections in horses (Fayer and Dubey, 1987). Positive reaction during an indirect hemagglutination test involving *S. cruzi* antigen where 9 out of 10 horses, all previously identified with EPM organisms in their tissues, tested positive for *S. cruzi* antibodies (Fayer and Dubey, 1987). These findings further aided in identifying *Sarcocystis* spp. as the parasite involved with EPM.

Additional research to suggest that the infectious agent for EPM was a *Sarcocystis* or a similar genus utilized samples from 25 known cases of EPM and tested them using anti-*S. cruzi* serum and anti-*T. gondii* serum. This immunohistochemistry work revealed that the protozoa, previously seen through H & E staining, reacted to the anti-*S. cruzi* serum but not to the anti-*T. gondii* serum (Granstrom et al., 1991).

The etiological agent of EPM, *Sarcocystis neurona*, was first identified in 1991 (Dubey et al., 1991). Structural studies of the parasite were performed from samples obtained from multiple cases of EPM from several different locations. It was determined that a single parasitic agent was present in all of these cases (Dubey et al., 1991). The parasite was cultured from a naturally infected horse for the first time in 1991; the horse had been given corticosteroids to increase the chance of isolating the parasite (Dubey et al., 1991; Dubey et al., 2001c). The name *Sarcocystis neurona* was proposed as the causative agent due to the localization of the organism in the horse (Dubey et al., 1991). The schizonts were only located within the spinal cord and brain of the horse, which is uncharacteristic of *Sarcocystis* species because they are normally found throughout the entire body (Dubey et al., 1991). The schizonts that were obtained from the infected
horses reacted positively to the *S. cruzi* antiserum, which assisted in identifying *Sarcocystis* as the parasitic species (Dubey et al., 1991). Further support that the parasite was *Sarcocystis* spp. included merozoites that were typical of *Sarcocystis* because they were located free in the host cell cytoplasm, lacked rhoptries, divided through endopolygeny, and were found in multiple different cells (Davis et al., 1991a). *S. neurona* was isolated from the spinal cord of two horses from California and similar conclusions were obtained regarding the identifications of the parasite when developed in bovine monocyte cell cultures (Davis et al., 1991a; Davis et al., 1991b).

Cases of EPM are primarily reported in North, Central and South America (MacKay, 1997). Cases that have been identified outside of the Americas have been isolated cases of horses that originated from the Americas. Due to the geographic localization of the disease, the proposed definitive hosts were limited to those endemic to the western Hemisphere. The raccoon, skunk, and opossum were originally suggested as possible definitive hosts due to their geographic distributions and their common association with horse farms (Granstrom et al., 1994). Only the skunk had specific antibodies toward *S. neurona* when serum from all three species was analyzed and therefore considered as a possible definitive host for *S. neurona* (Granstrom, 1995).

To help determine the definitive host of this parasite, the small subunit ribosomal RNA (SSURNA) gene of cultured merozoites of *S. neurona* was compared to several related species. These species included *S. muris, S. cruzi, Toxoplasma gondii* and *Cryptosporidium parvum*. These studies determined which region was unique to *S. neurona* to develop a species specific amplification primer (Fenger et al., 1995). Sporocysts were collected and analyzed, using this primer, from raccoons, opossums,
cats, skunks, a coyote and a hawk with the expectation that a common sequence could be identified between these sporocyst gene sequences and gene sequences from cultured *S. neurona* merozoites (Fenger et al., 1995). The opossum was highly suspected as the definitive host for *S. neurona* when the gene sequence from the opossum sporocysts divulged a 99.89% similarity with the gene sequence from the cultured *S. neurona* merozoites (Fenger et al., 1995).

Antemortem diagnosis of EPM became possible when a Western blot (Immunoblot) test specific for detecting *S. neurona* antibodies in the serum and cerebrospinal fluid (CSF) of horses was developed (Granstrom et al., 1993). Cell culture-grown merozoites are used as antigen, and the blots are probed with either test serum, CSF or both. Post-mortem tissue identification of EPM has been the only test possible for a definitive diagnosis but the development of the Western blot technique allows antemortem testing to help conduct serological surveys and identify exposure levels and environmental contamination.

Due to the development of an antemortem test, infection trials could be conducted to aid in the determination of the definitive host. In 1997, a major breakthrough occurred when researchers fed pooled feral opossum sporocysts to naïve foals and these foals not only showed clinical signs typical of EPM but also seroconverted and had lesions consistent with those seen in naturally affected horses (Fenger et al., 1997). However, *S. neurona* was not demonstrated histologically or by cell culture assay (Fenger et al., 1997). This research helped establish that the opossum was a definitive host for *S. neurona*, but because these opossums carried *S. falcatula*, *S.*
neurona and S. speeri sporocysts and the organism was not isolated histologically, it was unclear which Sporocyst species was the infectious agent (Fenger et al., 1997).

*S. falcatula* and *S. neurona* have similar sequence homology, and both use opossums as their definitive hosts. The first research model to differentiate which sporocyst species caused encephalitis used nude mice (Marsh et al., 1997a). *S. neurona* merozoites were inoculated into nude mice, which were then euthanized and tissue was harvested from multiple locations including the brain and the liver (Marsh et al., 1997a). Immunohistochemistry of the brain and liver tissue revealed *S. neurona* merozoites, demonstrating the neurologic infectivity of *S. neurona* (Marsh et al., 1997a). Dubey and Lindsay also provided evidence supporting that opossums were the definitive host for *S. neurona* and that *S. neurona* was the causative agent of EPM (Dubey and Lindsay, 1998). Their model used interferon-gamma gene knockout (KO) mice fed sporocysts from opossums, these mice developed neurological signs similar to those seen in horses with EPM (Dubey and Lindsay, 1998). *S. neurona* was identified in tissues of these mice using immunohistochemistry and the parasite was recovered in cell culture. Merozoites from this cell culture were then inoculated into more KO mice and produced encephalitis in these mice (Dubey and Lindsay, 1998).

Epidemiological studies were useful in identifying patterns and relationships between horses affected with EPM. Early studies regarding protozoal myelitis identified that young Standardbreds had an increased incidence of protozoal myelitis compared to Thoroughbreds (Rooney et al., 1970; Mayhew et al., 1976). Further research confirmed that Standardbreds had a higher incidence of exposure then Thoroughbreds but also concluded that the highest number of cases occurred in the spring and summer (Mayhew
and De Lahunta, 1978). However, epidemiologic research conducted by Boy et al showed that while 50% of horses affected were Standardbreds, the hospitalized cases of Standardbreds during a given period, was only 30% (Boy et al., 1990). In 1978, Cornell Veterinary College reported that 25% of neurologic cases seen at the university were due to EPM (Mayhew and De Lahunta, 1978).

Through epidemiological work, many factors that either increased or decreased the risk of developing the disease have been reported. One of the most significant factors identified to be a risk for the development of EPM was age. In 2000, Saville et al showed that 1-5 year olds were at great risk, while Boy et al found 1-6 year olds to be most commonly affected (Boy et al., 1990; Saville et al., 2000). Boy et al also suggested that sex was a determining factor with 43% of reported cases were stallions, but the stallion population hospitalized at this time was only 28% (Boy et al., 1990). Research conducted at the University of Pennsylvania reported 88% of EPM cases occurred in horses between 1-6 years of age. This research also showed a statistically significant increase in the incidence of EPM in Standardbreds (Divers, 1988). Fayer et al reported that 60% of histologically confirmed cases of EPM occurred in horses 4 years old or less (Fayer et al., 1990).

2.2. DIFFERENTIATION OF SARCOCYSTIS SPP

*Sarcozystis falcatula, Sarcozystis speeri and Sarcozystis neurona* all use the opossum as their definitive host (Box et al., 1984; Dubey et al., 1991; Fenger et al., 1997; Dubey and Lindsay, 1999). Because of the close molecular similarities in the ssuRNA genes of *S. neurona* and *S. falcatula*, in 1995 there was some confusion regarding the role
S. falcatula uses birds as intermediate hosts and the opossum as the definitive host (Dubey et al., 2001c). When S. falcatula was inoculated into both nude and interferon gamma KO mice no adverse effects were seen in either species (Dubey and Lindsay, 1998). Neither S. neurona nor S. speeri are infectious to budgerigars, however, S. speeri produced sarcocysts in both nude and interferon gamma KO mice, while S. neurona did not produce sarcocysts in either breed of mice (Dubey and Lindsay, 1999).

The highly conserved region of the 18ssu rRNA unit, small subunit ribosomal RNA (SSURNA), is often used in species differentiation and phylogenetic analysis (Hillis and Dixon, 1991). Preliminary research regarding differentiating S. falcatula and S. neurona found that the SSURNA were identical between the two species (Dame et al., 1995). Researchers extracted DNA from S. falcatula sarcocysts found in the muscle of a brown-headed cowbird (Molothrus ater) and from schizonts in fixed lung specimen of a Moluccan cockatoo (Cacatua moluccensis). These segments from the SSURNA gene were amplified, sequenced and compared with the SSURNA sequence from 2 known isolates of S. neurona merozoites (Dame et al., 1995). There was identical segment sequencing between the SSURNA of S. falcatula and the UCD1 isolate of S. neurona and only a 3 position difference between S. falcatula and the SN5 isolate of S. neurona thus suggesting that S. neurona and S. falcatula are synonymous (Dame et al., 1995). This research was the first to suggest that S. neurona and S. falcatula were identical.

A great deal of research was inspired, due to these results, to demonstrate the biological differences between S. falcatula and S. neurona. Some researchers focused on identifying structural and developmental differences between the two species (Lindsay et
al., 1999). Results of this research showed that *S. neurona* required 3 days for merozoites to undergo schizogony and these schizonts were smaller in size than *S. falcatula* which required 4 days until the onset of schizogony (Lindsay et al., 1999).

Additional evidence was provided that *S. falcatula* and *S. neurona* were separate species when Cutler et al demonstrated that horses fed sporocysts of *S. falcatula* failed to seroconvert to *S. neurona* antigens or show clinical signs (Cutler et al., 1999). All four of the experimentally inoculated horses failed to develop any clinical signs and remained negative when the serum and CSF were antigenically tested 12 weeks post inoculation (Cutler et al., 1999). This highly suggested there were differences between the species.

Further evidence suggesting biological differences between *S. falcatula* and *S. neurona* was demonstrated when laboratory raised budgerigars were experimentally challenged (Marsh et al., 1997b). Merozoites and schizonts from an isolate of *S. falcatula* were used for an in vivo inoculation of budgerigars while merozoites and schizonts of *S. neurona*, cultured from an EPM positive horse, were inoculated in additional budgerigars to compare the biological infectivity of each species (Marsh et al., 1997b). The budgerigars that were inoculated with *S. falcatula* died of pulmonary sarcocystosis while the budgerigars that were inoculated with *S. neurona* developed neither clinical signs nor developed histological lesions. Additional research indicating antigenic differences between the two species utilized polyclonal antiserum, which had been developed to react against *S. neurona* merozoites, showed no reaction to *S. falcatula* merozoites, tissue cysts or sporozoites (Marsh et al., 1997b).
Koch’s postulates were fulfilled and thus proved that *S. neurona* was a different species than *S. falcatula* when Dubey and Lindsay fed mice sporocysts from naturally infected opossums and those fed *S. neurona* sporocysts developed encephalitis and those fed *S. falcatula* sporocysts did not (Dubey and Lindsay, 1998). Homogenate of the liver, brain and spleen were used in cell culture and *S. neurona* was successfully recovered. The mice inoculated with *S. falcatula* merozoites and sporocysts did not become infected and no parasite was cultured from these mice (Dubey and Lindsay, 1998).

Another species, later identified as *Sarcocystis speeri*, was recognized when protozoa found in tissues of interferon gamma KO mice did not react with anti-*S. neurona* antibodies (Dubey et al., 1998; Dubey and Lindsay, 1999). Sporocysts from opossums were fed to budgerigars, nude mice and gamma-interferon KO mice (Dubey et al., 1998). Sporocysts from *S. neurona* and *S. falcatula* reacted as previously described (Dubey et al., 1998). The third sporocysts set seen were infective to KO and nude mice, had schizonts and merozoites that were found in multiple tissues but predominantly seen in the liver; however, these schizonts did not react to anti-*S. neurona* serum (Dubey et al., 1998). These merozoites were found to be ultrastructurally distinct from *S. neurona* and *S. falcatula* merozoites (Dubey et al., 1998). Sarcocysts were found in the leg muscles of 2 of these mice inoculated with the unknown *Sarcocystis* species. These sarcocysts had steeple-shaped protrusions on their cyst walls and were distinct from the sarcocysts of any other species of *Sarcocystis* (Dubey et al., 1998). This third species of *Sarcocystis* that utilizes the opossum as their definitive host was later identified as *Sarcocystis Speeri* (Dubey and Lindsay, 1999).
Prior to the development of specific DNA markers to help distinguish the different Sarcocystis species, random-amplified polymorphic DNA techniques were utilized to amplify DNA from isolates of S. neurona and S. falcataula (Tanhauser et al., 1999). Specific DNA sequences were then created and utilized to design PCR primers to amplify specific Sarcocystis spp. This made it possible to identify sequence heterogeneity and allowed for the characterization of isolates from opossum feces (Tanhauser et al., 1999). Due to the sensitivity of this test it is possible to detect a minimum amount of parasite in a given CSF sample, however, since the test focuses on the detection of circulating merozoites, the lack of these creates issues regarding the certainty of this test (Marsh et al., 1996).

In an attempt to further compare the genetic relationship between S. neurona and S. falcataula isolates, researchers utilized a rapidly evolving genetic marker of rRNA called ITS-1 (Marsh et al., 1999). The internal transcribed spacer region 1 (ITS-1) of rRNA has been described as a suitable region to be used to identify species level differences (Hillis and Dixon, 1991). ITS-1 region sequences from multiple isolates of S. falcataula were compared to a full sequence of an S. neurona isolate obtained from a horse diagnosed with EPM (Marsh et al., 1999). Aligned sequences of S. neurona and S. falcataula revealed a consistent 12 nucleotide difference, thus suggesting that the ITS-1 region can be used to distinguish S. neurona from S. falcataula (Marsh et al., 1999).

2.3. SARCOCYSTIS NEURONA MIGRATION

The first model that categorized the migration and development of S. neurona utilized gamma interferon knockout mice that were fed S. neurona sporocysts (Dubey,
This experiment utilized 50 gamma interferon knockout mice that were fed graded doses of *S. neurona* sporocysts and euthanized at differing time periods over the next 62 days (Dubey, 2001). In mice that were euthanized within 1-3 days PI (post inoculation), the organism was primarily identified within the intestines. In mice euthanized between 4-11 days PI, the organisms were seen in several visceral tissues (Dubey, 2001). Beginning with day 13 PI, schizonts and merozoites were present in sections of the brain of all inoculated mice. All areas of the brain were parasitized but the hind brain was more severely affected (Dubey, 2001). Of the 28 mice whom demonstrated infection and that were euthanized between 20-62 days PI, there were organisms found in the brain of all 28, the lungs of 14, the hearts of 8, and the eyes of 3. The amount of organisms found in tissues outside of the brain was minimal when compared to the organisms found within the brain. There were no sarcocysts found in any of the mice (Dubey, 2001). Based on these results, researchers suggested that *S. neurona* is initially transported through the lymphatic circulation (Dubey, 2001).

The first study to successfully show the early migration of *S. neurona* in the horse utilized a model involving 6 ponies inoculated with *S. neurona* sporocysts (Elitsur et al., 2007). These ponies were orally inoculated with sporocysts and then euthanized at varying intervals over the next 9 days. Results revealed parasite isolated from mesenteric lymph nodes of ponies euthanized 1, 2, and 7 days PI, the liver of ponies euthanized 2, 3, 5, and 7 days PI, and the lungs of ponies euthanized 5, 7, and 9 days PI (Elitsur et al., 2007). *S. neurona* was isolated from tissues of all the ponies by bioassay in knockout mice and isolated through cell culture from 2 of the ponies (Elitsur et al., 2007). None of the ponies at the time of euthanasia demonstrated any clinical signs associated with EPM,
such as neurologic deficits (Elitsur et al., 2007). However, ponies euthanized at 7 and 9 days PI showed foci of inflammation commonly associated with EPM when tissue samples were examined histologically, but *S. neurona* specific IHC staining on tissue with these lesions revealed no parasite (Elitsur et al., 2007). Due to the isolation of the parasite from the liver and lung, researchers suggest that there is a hematogenous dissemination of the parasite as well as a lymphatic dissemination due to the isolation of the parasite from the mesenteric lymph nodes (Elitsur et al., 2007).

### 2.4. LIFE CYCLE OF SARCOCYSTIS NEURONA

#### 2.4.1 Sexual Reproduction

*Sarcocystis* species typically have a two host predator-prey or scavenger-carrion life cycle (Dubey et al., 1989). The normal Apicomplexan life cycle involves three stages of development: gametogony, sporogony, and schizogony (Dubey et al., 1989). The sexual stage consisting of gametogony and asexual stage of sporogony occur in the definitive hosts (predator or scavenger) and leads to the production of sporocysts. Schizogony, asexual stage of reproduction, occurs in the intermediate hosts (predator or carrion) and results in sarcocyst formation (Dubey et al., 1989).

The definitive hosts normally ingest sarcocyst infected tissues from an intermediate host and then produce sporocysts. The intermediate host acquires the sporocyst, most often by ingesting them, and then develops sarcocysts. For the life cycle to be perpetuated in nature, both the definitive and intermediate hosts must be present.

The definitive host for *S. neurona* has been established as the opossum (*Didelphis virginiana*) (Fenger et al., 1997; Dubey and Lindsay, 1998). Following the death of the
intermediate host, sarcocysts can remain viable for extended periods of time thus allowing the opossum to ingest infected muscle tissues (Odening, 1998). The sexual phase of *S. neurona* is comparable to other *Sarcocystis* species (Lindsay et al., 2004). The definitive host (the opossum), becomes infected by ingesting muscular or neural tissue containing mature sarcocysts. Bradyzoites are released from the sarcocysts by digestion in the stomach and intestines (Dubey et al., 1989). The bradyzoites quickly penetrate the mucosa of the small intestine and transform into male (micro) and female (macro) gamonts. The microgametes actively move to the periphery of the macrogamont and fertilization occurs. After fertilization a wall is formed around the zygote and the oocyst is created (Dubey et al., 1989). The sexual process of gametogony is rapid leading to the production of a fertilized, unsporulated oocyst (Box and Duszynski, 1980; Dubey et al., 1989). The process of gametogony and fertilization is asynchronous, thus allowing gamonts and oocysts to be found at the same time (Dubey et al., 1989). The oocyst sporulate in the lamina propria, this produces two sporocysts each containing 4 sporozoites (Levine, 1986; Dubey et al., 1989). The oocyst wall is thin and will often rupture, thereby releasing sporocysts into the intestinal lumen and finally passed in the feces (Dubey et al., 1989).

Sporogony occurs as sporocysts pass through the small intestines leading to the maturation of the sporocysts and development into the infective sporozoites stage (Dubey et al., 1989). Four sporozoites form in each sporocyst. It is difficult to identify all four simultaneously due to their arrangement when viewed microscopically (Dubey et al., 1989). Once sporocysts are released into the environment, they are infective and can remain so for long periods of time as well as through diverse climate conditions.
The prepatent and patent period for *Sarcocystis* species varies but is typically seen between 7 and 14 days post ingestion of the sarcocysts, however extended patent periods have been seen with some *Sarcocystis* species (Dubey et al., 1989). Delayed development was ruled out as a reason for prolonged shedding, when studies showed that completely mature sporocysts were found on the same day post infection as gamonts (Box and Duszynski, 1980). The extended patent periods are probably due to intestinal contractions which will cause the sporocysts to be extricated from the lamina propria, the sporocysts are then forced from the villi into the small intestines and eventually expelled into the environment (Box and Duszynski, 1980; Dubey et al., 1989).

### 2.4.2 Asexual Reproduction

Intermediate hosts of *Sarcocystis* species become infected by ingesting sporocysts either in food or in water (Dubey et al., 1989). The sporozoites rapidly excyst from the sporocysts, under experimental conditions. *S. neurona* sporozoites were shown to excyst within 15 minutes (Dubey et al., 1989; Lindsay et al., 2004). Once the sporozoites are released they begin development within the intestinal epithelium, with the first round of merogony occurring normally within the endothelial cells located in arterial vessels (Dubey et al., 1989). *Sarcocystis* schizonts multiply by a process known as endopolygeny where merozoites begin to develop and bud in a rosette pattern around a central schizont (Long, 1990; Dubey et al., 2003). A second occurrence of merogony occurs in the endothelium of capillaries, as well as small arteries following the release of merozoite from schizonts in the blood stream (Gardiner et al., 1988; Dubey et al., 1989).
The type of host cell and the number of generations of schizogony vary depending on the species of *Sarcocystis* (Dubey et al., 1989). Merozoites that are released from the terminal generation of schizogony initiate the sarcocyst formation. The intracellular merozoites are surrounded by a parasitophorous vacuole and become round to ovoid in shape which are then called metrocytes (Dubey et al., 1989). The parasitophorous vacuole then differentiates into a sarcocyst wall (Long, 1990). After repeated division of the metrocytes, the sarcocyst is filled with bradyzoites which signals that it is in an infectious stage for the predator or scavenger, while immature sarcocysts and schizonts are not infectious for the definitive host (Dubey et al., 1989).

Sarcocysts may begin to disappear after 3 months postinoculation, or they may remain present for the duration of the intermediate host’s life (Dubey et al., 1989). Sarcocysts generally become infectious around day 75 post inoculation, but this is also variable between species of *Sarcocystis* (Dubey et al., 1989). The severity of infection has been associated with the proportion of sporocysts ingested as well as the immunocompetency of the animal. It has been determined that smaller doses of sporocysts are needed to cause infection in smaller intermediate host species (Dubey et al., 1989; Long, 1990).

2.5. **SURFACE ANTIGENS OF SARCOCYSTIS SPECIES**

One of the first studies examining the presence of surface antigens associated with *Sarcocystis* species examined *Sarcocystis muris* zoite extracts recovered from infected muscle tissues of mice (Abbas and Powell, 1983). Antigens were tested via agar gel diffusion and immunoelectrophoresis against sera from infected mice. The SDS-PAGE of
the extracts revealed 8 detectable polypeptides that were then labeled against the parasite surface antigens using immune sera. The Immunoprecipitates revealed three antigens of varying molecular weights (Abbas and Powell, 1983). Sporozoites and culture derived merozoites of *Sarcocystis cruzi* from mice were found to have similar reacting surface antigens during detection by immunofluorescence (Burgess et al., 1988). At least one surface epitope of the sporozoites and merozoites also reacted with antigens of bradyzoites during western blot analysis, while others showed no reaction. This indicated that epitopes from these three stages of *Sarcocystis* maybe either distinct or similar (Burgess et al., 1988). Cross reactivity of surface proteins between two developmental stages (cyst merozoites and sporozoites) of *S. muris* and *S. suicanis* was demonstrated using immunoblots and gel electrophoresis (Sommer et al., 1992).

Major surface proteins were identified from *Sarcocystis muris* and *Sarcocystis suicanis* using SDS-PAGE with Western blot analysis and indirect fluorescent antibody tests (IFAT) (Sommer et al., 1991). Molecular mass of 31 kDa for *S. muris* and 33 kDa for *S. suicanis* responded strongly to reactions designed to label surface proteins (biotinylation and radioiodination of intact organisms) (Sommer et al., 1991). That these proteins represented immunogenic surface antigens was supported by results from immunoassays executed with mono and polyclonal antibodies raised against these proteins (Sommer et al., 1991). Indication that the similarities of surface antigens between apicomplexan parasites may exist was demonstrated when surface antigen 2 (SAG2) for *Toxoplasma gondii* was evaluated with the surface antigen of *S. muris* (SnSAG1) (Cesbron-Delauw, 1995). Even though the similarities between the two SAGs were minimal it was thought to have functional implications which may help in
understanding molecular relationships between closely related coccidian parasites (Cesbron-Delauw, 1995). Using reverse transcription PCR (RT-PCR) and restriction fragment length polymorphism (RFLP) methods, the surface antigen 1 (SAG1) isolates of S. neurona and S. neurona-like parasites were compared to SAG1 gene from isolates of Neospora species (Hyun et al., 2003). The SAG1 gene varied from 73 to 100% between isolates of S. neurona parasites and 96 to 98% between isolates of Neospora parasites. This indicated that there was a strong conservation of the cysteine residue between isolates of S. neurona and suggested that there are not multiple SAG1 genes that are preferentially expressed at a given time for a single isolate (Hyun et al., 2003).

*Sarcocystis neurona* surface antigen 1 (SnSAG1) was identified from a 26kDa protein that was isolated and amplified from culture derived S. neurona merozoites (Ellison et al., 2002). SnSAG1 is recognized on immunoblots when probed with intrathecal antibodies from a clinically diagnosed EPM horse infected with S. neurona (Ellison et al., 2002). Immunofluorescent antibody assays and post-embedding immunogold labeling studies localized SnSAG1 on the surface of S. neurona merozoites (Ellison et al., 2002). In 1 study it appeared that S. neurona is able to induce immunosuppression toward parasite-derived antigens (SnSAG1) as parasite-specific response decreased (Spencer et al., 2004). This study showed the antigenic response to SnSAG1 between EPM-negative horses and EPM-positive horses. The negative horses showed a very significant response to SnSAG1 yet the EPM-positive horses did not show a high response to SnSAG1 (Spencer et al., 2004).

The designation of *S. neurona* proteins SnSAG1, SnSAG2, SnSAG3, and SnSAG4 was due to their surface localization and homology to *T. gondii* surface antigens.
This was based on the analysis of *S. neurona* expressed sequence tag (EST) database that revealed four paralogous proteins that exhibited homology to the family of surface antigens of *T. gondii*. The primary peptide sequence of *S. neurona* proteins had a two-domain structure (similar to that seen in *T. gondii* SAG proteins) and each contained an amino-terminal signal peptide and a carboxyl-terminal glycolipid anchor addition site, which indicates surface localization (Howe et al., 2005). All four of the proteins were found to be membrane associated and present on the surface of *S. neurona* merozoites. The SnSAGs ability to elicit a strong immune response in infected and immunized animals and their conserved structure suggests that SnSAGs serve as adhesions for attachment to host cells (Howe et al., 2005).

### 2.6. THE OPOSSUM, DEFINITIVE HOST OF *SARCOCYSTIS NEURONA*

#### 2.6.1 Life Style and Habits of the Opossum

As North Americas only marsupial, the opossum is unique and is marked by its narrow tapered snout, jaws that contain fifty teeth, grayish fur and prehensile tail (Krause and Krause, 2006). They range in size from 1.8 to 5.5 kilograms (Krause and Krause, 2006). The current distribution of the opossum is seen throughout the majority of the United States with the exception of some of the northern and western states(Krause and Krause, 2006). This distribution of opossums is similar to the distribution of EPM and areas with lower seroprevalence of *S. neurona* appear to be outside the natural range of the opossum(Blythe et al., 1997). Opossums are not considered territorial necessarily, but they are solitary and will exclude other individuals from their area. The size of their territory is variable and dependent on the availability of food(Krause and Krause, 2006).
While the opossum prefers deciduous woodlands they are highly adaptable and can often be found in prairies, marshes and farmlands (Gardner, 1982; Krause and Krause, 2006). The opossum is very adaptable to human habitations and is often found in close proximity to domestic animals, sharing in their feed and living spaces (Krause and Krause, 2006). While the opossum is a nocturnal animal, during the cold winter months they remain active and will often come out during the day to scavenge for food before returning to a den, hollow tree or brush for the remainder of the day (Reynolds, 1945; Krause and Krause, 2006).

Opossums are considered opportunistic, year round feeders that will eat just about anything that is available (Gardner, 1982). It has been stated that the opossum is a carnivore by preference and an omnivore by necessity (Brocke, 1970). Since the opossum is considered an omnivore, eating a vast array of plants, insects, berries and garbage they are also scavengers that eat a good deal of carrion. Their diet is variable, depending on their environment as well as the season (Krause and Krause, 2006). They are known for their exceptionally poor eyesight and use their keen sense of smell to locate their food (Krause and Krause, 2006).

Because the opossum’s diet is so varied it is extremely difficult to ascertain if they have any predilection for food, in particular small mammals. There have been multiple studies conducted in an attempt to identify the components of the opossum’s diet. Some of the studies focused on examining the contents of the stomach, which is a more definitive investigation, but most focused on analyzing fecal content. These studies have furthered our understanding of the opossum’s food preferences, and have assisted in identifying the intermediate hosts associated with this disease.
2.6.2 Species of Opossums and Their Role in the Perpetuation of Sarcocystis species

*Didelphimorphia* is the order, which contains the new world opossums. There are six members of the genus *Didelphis*. Five members of these species are found in South and Central America; *D. albiventris*, *D. aurita*, *D. imperfecta*, *D. marsupialis*, and *D. pernigra*, with the sixth member of the species *Didelphis virginiana* the only species seen in the United States and southern Canada (Gardner, 1982; Feldhamer et al., 2003). Opossums have been identified as the intermediate host of *S. greineri* and the definitive host for several *Sarcocystis* species including *S. neurona*, *S. falcatula*, *S. speeri*, *S. lindsayi* and other as yet unnamed species (Elsheikha et al., 2004a).

The South American opossums *D. albiventris* and *D. marsupialis* as well as *D. virginiana* have been identified as the definitive hosts for *S. speeri* and *S. falcatula* (Dubey and Lindsay, 1999; Dubey et al., 1999; Dubey et al., 2000a; Dubey et al., 2000c). The distribution of EPM in South America supports the theory that South American opossums are capable of shedding *S. neurona* sporocysts. Preliminary studies establishing phylogenetic similarities between the South American opossum and the North American opossum showed the successful transmission of *S. speeri* from *D. albiventris* to *D. virginiana* by feeding infective sarcocysts (Dubey et al., 2000d). This was done by the collection of sporocysts from a *D. albiventris* that were then inoculated in KO mice that developed sarcocysts. Infective mouse tissues were then fed to *D. virginiana*, which began to shed sporocysts which were determined to be *S. speeri* based on the morphology and antigenicity (Dubey et al., 2000d).
While the North American opossum *D. virginiana* had previously been identified as the definitive host of *S. neurona*, it wasn’t until 2001 that it was established that the South American opossum *D. albiventris* was also capable of transmitting *S. neurona* (Dubey and Lindsay, 1998; Dubey et al., 2001b). *S. neurona* sporocysts were isolated from two of eight opossums (*D. albiventris*) from Brazil. Sporocysts from these two opossums were fed to interferon gamma KO mice, which then developed neurologic sarcocystosis. Through immunohistochemical staining with anti-*S. neurona* antibodies, *S. neurona* merozoites were demonstrated in the brain tissue of these mice (Dubey et al., 2001b). The parasite was then cultivated in cell culture, and *S. neurona* DNA was isolated from cultured merozoites (Dubey et al., 2001b).

### 2.7. CLASSIFICATION OF SARCOCYSTIS SPOROCYSTS

*Sarcocystis neurona* is unique because the molecular means of diagnosis were developed for its sporocysts before its life cycle was revealed (Tanhauser et al., 1999; Lindsay et al., 2004). While the opossum is the definitive host for *S. neurona* it is also known to be the definitive host for at least 4 other species of *Sarcocystis*. These early molecular diagnostics would later prove extremely helpful in determining the natural intermediate hosts for *S. neurona* (Lindsay et al., 2004).

#### 2.7.1 Viability of Sporocysts

Sporocysts are produced during the sexual stage of development. With minor variations in size, all *Sarcocystis* sporocysts and oocysts are structurally similar (Dubey et al., 1989). Sporocysts are encapsulated by a double membrane wall which assists in environmental viability and sporozoite formation (Dubey et al., 1989). Since the
sporocysts of *Sarcocystis* are sporulated and small, they are resistant to many environmental factors. They are therefore able to disperse in the environment which has significant epidemiological importance (Savini et al., 1996).

The viability of multiple *Sarcocystis* species has been studied. Studies involving sporocysts from *S. cruzi* were able to maintain their viability at temperatures ranging from 4°C to 37°C and under multiple conditions of relative humidity (Savini et al., 1996). Earlier studies involving *S. ovicanis* showed that sporocysts are resistant to freezing using sporocysts isolated from feces from a frozen coyote carcass, which was then thawed and fed to sheep. These sheep then developed a *Sarcocystis* infection (Leek, 1986). While sporocysts have been shown to maintain their viability for long periods of time during extreme climate conditions, when a normal temperature and humidity are implemented, they have a shortened viability time (Savini et al., 1996). Under moderate temperature and humidity, sporocysts of *S. gigantea* were able to remain viable for the longest period of time (McKenna and Charleston, 1994). Another study examining the effects of temperature on *Sarcocystis* species, used buffalo cardiac muscle containing *S. levinei* that was fed to dogs (Srivastava et al., 1986). Tissues that were heated between 65-75 degrees C did not shed sporocysts, while tissues heated between 40 and 60 degrees C did shed sporocysts. Dogs infected with tissues stored at -4 degrees C for 48 hours did not shed sporocysts, but those fed infected tissues that were stored at -2 degrees C for 24 hours did shed sporocysts (Srivastava et al., 1986).

Studies that test the viability of *S. neurona* sporocysts using moist heat revealed that sporocysts heated to 50°C for 60 min and 55°C for 5 min were infective when inoculated into KO mice (Dubey et al., 2002). This study also showed that sporocysts
were heated to 55°C for 15 min and 60°C or more for 1 min the sporocysts were rendered noninfective to KO mice (Dubey et al., 2002). This study also tested the viability of sporocysts with disinfectants showing the sporocysts remained viable when treated with bleach (10, 20, and 100% concentrations), 2% chlorhexidine, 1% betadine, 5% o-benzyl-p-chlorophenol, 12.56% phenol, 6% benzyl ammonium chloride, and 10% formalin. While treatment with undiluted ammonium hydroxide (29.5% ammonia) for 1 hr proved effective for killing sporocysts, using a 10 fold dilution (2.95% ammonia) for 6 hr did not result in killing sporocysts (Dubey et al., 2002).

Another study that examined the viability of *S. neurona* sporocysts used Gamma-interferon knockout mice (Cheadle et al., 2001c). In this study, sporocysts were collected from opossums (*D. virginiana*), processed and stored for varying periods of time. The mice were then orally inoculated with different isolates at different doses and at differing ages of the isolates (Cheadle et al., 2001c). Of the 17 mice used in the study, 15 developed signs consistent with neurologic disease, and within brain tissue of 13 of these mice, the organism was observed (Cheadle et al., 2001c). The base dose shown to be viable in this study was of 500 sporocysts of inoculums that was equal to or less than 7 months old. If the sporocysts used had been processed and stored for more than 16 months they were found to not be viable. This suggested that the viability of *S. neurona* sporocysts decreases significantly with extended storage periods (Cheadle et al., 2001c). This study confirmed that sporocysts determined to be *S. neurona* by molecular characterization could infect and cause disease in susceptible hosts. This is beneficial since the use of interferon gamma KO is timely and expensive, however it still remains the ideal way of determining the viability of sporocysts (Cheadle et al., 2001c).
Another study that examined the viability of *S. neurona* sporocysts after an extended storage was conducted using sporocysts that remained in storage from 1996 to 2002 and stored at 4°C (Elsheikha et al., 2004b). The viability was assessed using propidium iodine (PI) exclusion assays, in vitro excystation and development in tissue culture and bioassay in interferon gamma KO mice. Studies have used PI staining to differentiate between live (viable) and heat-killed (nonviable) *S. neurona* sporocysts (Elsheikha and Mansfield, 2004). PI is excluded from live sporocysts but penetrates compromised sporocyst membranes and stains sporozoite nuclei of dead sporocysts (Elsheikha and Mansfield, 2004). Sporocysts retained their ability to excyst after 7 years of storage at 4°C thereby showing long-term storage had no effect on the rate of excystation(Elsheikha et al., 2004b). The results of the PI exclusion assay, the decreased in vitro infectivity and development in cell culture, and the decreased infectivity of mice all indicated that the viability of *Sarcocystis* sporocysts decline as sporocysts age(Elsheikha et al., 2004b).

### 2.7.2 Prevalence of *Sarcocystis* Species in Opossums

For *S. neurona* to infect an intermediate or aberrant host, the host must consume infected sporocysts. Early understanding of distribution of the disease focused on the distribution of the definitive host, the opossum. Since the opossum is the definitive host for multiple species of *Sarcocystis* it was important to understand the prevalence of each species to help predict where disease was most likely to be seen. In one study using naturally infected opossums, 24 of 44 intestinal scrapings tested positive for the presence of sporocysts (Dubey, 2000). The geographic distribution of opossums included
Louisiana, Florida, Pennsylvania, Maryland, and Virginia. Sporocysts from 23 of these scrapings were given to budgerigars, with 21 samples demonstrating infectivity and indicating that *S. falcatula* was present. Sporocysts collected from all 24 positive scrapings were given to interferon gamma KO mice or nude mice, with 14 of the 24 samples indicating infectivity (Dubey, 2000). *S. neurona* was detected when tissues from KO mice fed inocula from 8 opossums tested positive using anti-*S. neurona* antibody staining techniques and also, when cultured parasite from the brains of mice were found (Dubey, 2000). *S. speeri* was identified when tissues of KO mice fed inocula from 8 opossums, stained positive with anti-*S. speeri* antibodies. These results showed a prevalence of 47.7% for *S. falcatula*, 18.1% for *S. neurona*, and 18.1% for *S. speeri*. *S. neurona* was found alone in 1 opossum, and *S. speeri* was also found alone in 1 opossum. Mixed *Sarcocystis* infections were present in 21 opossums (Dubey, 2000).

A similar study conducted in Mississippi indicated that gastrointestinal scraping from 24 of 72 wild caught opossums indicated presence of *Sarcocystis* sporocysts. Of these 24 scrapings, 19 of them tested positive for *S. neurona* (Dubey et al., 2001a).

Another study conducted in Michigan using gastrointestinal scrapings from 206 native opossums resulted in 31 of 206 (15%) testing positive for *S. neurona* sporocysts (Elsheikha et al., 2004a). A study conducted in California indicated that 5.9% (17 of 288) of opossums tested positive for *S. neurona* when gastrointestinal scrapings and feces were analyzed (Rejmanek et al., 2009). This study also showed a statistical increase in the prevalence of *S. neurona* from opossums located in the Central Valley when compared to opossum closer to the coast (Rejmanek et al., 2009). These high prevalences of *S. neurona* sporocysts indicate that the opossum act as an ample reservoir
of infection to other animals throughout the United States (Dubey, 2000; Dubey et al., 2001a; Elsheikha et al., 2004a; Rejmanek et al., 2009).

2.7.3 Evaluation of Sarcocystis sporocysts size and shedding

The size and morphologic characteristics of isolates of Sarcocystis shed by the opossum were examined in an effort to ascertain if there were any distinct differences that would be useful in discriminating between the isolates and species (Cheadle et al., 2001a). It was determined that S. neurona sporocysts were 10.7 µm x 7.0 µm, S. falcatula were 11.0 µm x 7.1 µm, and S. speeri were 12.2 µm x 8.8 µm (Cheadle et al., 2001a). The length and width of S speeri were statistically different from other sporocysts. The length of S. neurona and S. falcatula were statistically different from each other. However there were no consistent differences between taxa based on the internal structure of the sporocysts (Cheadle et al., 2001a).

To ascertain the amount of sporocysts shed by an experimentally infected opossum, researchers used 5 laboratory raised opossums that were fed muscle tissue from brown-headed cowbirds that contained sarcocysts of S. falcatula (Porter et al., 2001). Fecal flotation’s confirmed shedding of sporocysts by all five opossums. Opossums ingesting infected tissues began shedding sporocyst between 7 and 16 days (Porter et al., 2001). The number of sporocysts varied between opossums, but on average, the opossums shed 17.5 grams of feces which contained 1480 sporocysts per gram. This resulted that on average, 25,900 sporocysts/day were shed with a maximum of 647,500 sporocysts per day (Porter et al., 2001). Opossums continue to shed sporocysts until they were euthanized (46-200 DPI) with the maximum mean shedding occurring at 71 DPI.
Gastrointestinal tract analysis showed that the majority of the oocysts and sporocysts were found in clusters within the lamina propria of the luminal two-thirds of the villi found in the duodenum and jejunum, (Porter et al., 2001).

2.8. THE INTERMEDIATE HOSTS OF SARCOCYSTIS NEURONA

Sarcocysts form in the intermediate host following ingestion of infective sporocysts found in the food or water (Dubey et al., 1989). After ingestion and absorption the sporocyst undergoes multiple rounds of asexual reproduction until a sarcocyst is formed in the muscle tissue (Dubey et al., 1989). Intermediate hosts of Sarcocystis species tend to be herbivores and prey species, following the normal Sarcocystis predator-prey life cycle. Most Sarcocystis species have 1 or 2 intermediate hosts and 1 definitive host. S. neurona is unique since it has a large and diverse variety of intermediate hosts including domestic cats, skunks, nine-banded armadillos, sea otters, and raccoons (Butcher et al., 2001; Cheadle et al., 2001b; Cheadle et al., 2001d; Dubey et al., 2001d; Dubey et al., 2001e; Turay et al., 2002).

2.8.1 Cats as Intermediate Hosts

Most intermediate hosts of Sarcocystis species are herbivores, but there have been reports of cats, which are considered carnivores, being infected with sarcocysts. Normally felines are found to be definitive hosts for Sarcocystis species thereby the parasite only undergoes the sexual phase of reproduction in theses hosts (Dubey et al., 1989). Since cats are considered predators they do not follow the normal Predator –prey doctrine associated with most Sarcocystis species and therefore would not be considered ideal for the perpetuation of the parasite in nature (Greiner et al., 1989).
Cats that become infective with sarcocysts may suffer from an immunocompromising disease, there have been several reports of cats with sarcocyst infections developing severe illness (Kirkpatrick et al., 1986; Greiner et al., 1989). In one study examining the existence of sarcocysts in cats, 11 of 14 wild Florida panthers and 4 of 4 cougars developed sarcocysts in their tongues, skeletal muscles and diaphragms (Greiner et al., 1989). These results are very similar to results seen in *S. neurona* infected cats, which develop sarcocysts in their tongues, diaphragms, abdominal muscles, and thigh muscles (Kirkpatrick et al., 1986; Greiner et al., 1989; Dubey et al., 2000b). One of the animals, the Florida panther, is known to prey upon opossums (Greiner et al., 1989). Even though cats aren’t considered a predator species and an unlikely candidate for an intermediate host, sarcocysts are commonly found in cats without causing any apparent problem (Eisenstein and Innes, 1956).

The first research to demonstrate the cat as an intermediate host of *S. neurona* used sporocysts collected from a naturally infected opossum and orally inoculated them into the cat (Dubey et al., 2000b). The cat developed sarcocysts in the muscle tissue. These infected tissue samples were then fed to laboratory raised opossums which then proceeded to shed sporocysts in their feces. The sporocysts from the gastrointestinal scrapings were bioassayed in gamma interferon KO mice (Dubey et al., 2000b). *S. neurona* schizonts, merozoites and sporozoites were found in the tissues from KO mice. Ponies were also orally inoculated with sporocysts collected via gastrointestinal scrapings. They developed neurologic deficits consistent with EPM, and their sera tested positive for antibodies to *S. neurona* (Dubey et al., 2000b).
To determine if cats have antibodies that react to *S. neurona* similar to antibodies from horses with EPM, nine feral cats were analyzed (Turay et al., 2002). Two out of the nine cats showed positive antigen-antibody reactivity to *Sarcocystis* species, but only one of these samples reacted positively when tested against SN-MU1, a *S. neurona* antigenic strain (Marsh et al., 2001; Turay et al., 2002) Tissues from the one cat that possessed tissue sarcocysts in the tongue were fed to laboratory raised opossum. The opossum shed sporocysts, which were then collected via gastrointestinal scrapings and fed to gamma-interferon KO mice (Turay et al., 2002). Three of the mice developed neurologic signs. Via immunohistochemistry, histopathology, parasite isolation and molecular analysis, it was determined that mice were infected with *S. neurona* parasites. This supported previous evidence that cats can serve as an appropriate intermediate host for *S. neurona* (Turay et al., 2002).

The first study to use *S. neurona* merozoites to inoculate an intermediate host used five laboratory raised cats (Butcher et al., 2001). Cat 1 received $1 \times 10^7$ SN-UCD1 derived merozoites IV, cat 2 received $1 \times 10^7$ SN-Mucat2 derived merozoites IV, cat 3 received $5 \times 10^7$ SN-UCD1 derived merozoites divided equally over three routes of inoculation IV, IM, and SQ, and cat 4 received $5 \times 10^7$ Sn-Mucat2 derived merozoites divided equally over three routes of inoculation IV, IM, and SQ (Butcher et al., 2001). Cat 5 was used as a negative control and received $3 \times 10^7$ of host cells, equine dermal and deer testes. The 4 cats inoculated with *S. neurona* merozoites developed antibodies that reacted to *S. neurona* merozoite antigens, however tissue sarcocysts were only detected in the two cats that were inoculated with the cat derived Sn-Mucat2 merozoites (Butcher et al., 2001). Muscle tissues from all 4 cats were fed to laboratory raised opossums,
sporocysts were detected in gastrointestinal scrapings of the opossum that was fed muscle
tissue from the cat inoculated with $5 \times 10^7$ merozoites. These sporocysts were orally
inoculated into gamma-interferon KO mice, which developed clinical signs of
encephalitis (Butcher et al., 2001). This study showed that cat derived $S. \text{neurona}$
merozoites could be inoculated into cats and result in formation of tissue sarcocysts
(Butcher et al., 2001).

2.8.2 Skunks as Intermediate Hosts

Skunks have a large distribution that covers the majority of the United States,
Canada, and northern Mexico, which is similar to the distribution of EPM (Feldhamer et
al., 2003). The striped skunk is the definitive host for $Sarcocystis \text{rileyi}$ (Dubey et al.,
1989). Preliminary evaluation that established skunks can be infected with $S. \text{neurona}$,
showed that a protozoa found in the central nervous system of a skunk reacted positively
to $S. \text{neurona}$-specific antibodies using an immunohistochemical test (Dubey and Hamir,
2000).

In an effort to establish the skunk as an intermediate host for $S. \text{neurona}$,
laboratory raised skunks were orally inoculated with $5 \times 10^3$ and $5 \times 10^4$ sporocysts,
respectively, of an $S. \text{neurona}$ isolate collected from a naturally infected opossum
(Cheadle et al., 2001d). The skunks formed tissue sarcocysts. These tissues were then
fed to laboratory raised opossums which shed sporocysts in their feces. The sarcocysts
found in the skunks and sporocysts obtained from the opossum were identified as $S. \text{neurona}$ using PCR and DNA sequencing analysis (Cheadle et al., 2001d). The
sporocysts collected from the opossums were inoculated into naïve ponies and gamma-
interferon KO mice. The ponies developed antibodies to *S. neurona* when tested using an immunoblot analysis of their sera and CSF. The KO mice developed serum antibodies to *S. neurona* and showed clinical signs of encephalitis (Cheadle et al., 2001d). Also when CNS tissues were examined using immunohistochemical staining, *S. neurona* merozoites were detected in the tissues. This study established that the skunk is an intermediate host for *S. neurona* (Cheadle et al., 2001d).

A study conducted in Connecticut used 24 wild caught skunks and tested sera for the presence of *agglutinating* antibodies using the *S. neurona* agglutination test (SAT) and employing formalin-fixed merozoites as antigens (Mitchell et al., 2002). The prevalence of *S. neurona* antibodies in skunks was 46% (11/24) in this geographic region (Mitchell et al., 2002). Skunks have previously been shown to develop sarcocystis related encephalitis and recent studies have suggested that canine distemper virus may cause immunosppression and allow opportunistic *S. neurona* to develop in the CNS of skunks causing encephalitis (Burcham et al.).

### 2.8.3 Nine-banded armadillos as Intermediate Hosts

The nine-banded armadillo (*Dasypus novemcinctus*) is a very distinct mammal. Its name means “little armored one” due to the fact that its entire body is covered by armor with the exception of its underside (Burde and Feldhamer, 2005). The armadillo is a nocturnal animal and is considered omnivorous, feeding on insects, vegetation, birds and small vertebrates; and it is not considered a prey species (Burde and Feldhamer, 2005). However, they are often killed by automobiles thus making them an available food source for opossums (Cheadle et al., 2001b). The distribution of the armadillo has
expanded to include most of the southeastern United States, ranging throughout Texas, the Gulf Coast, and into Florida. It has been found as far north as Missouri and southern Illinois. The armadillo is sensitive to cold temperatures so farther expansion into northern territories is unlikely (Burde and Feldhamer, 2005).

Preliminary research relating the armadillo to Sarcocystis infection examined tongues of 24 armadillos collected between 1989 and 1994 from Texas, Oklahoma, Kansas, and Arkansas. 23 of the 24 armadillos had sarcocysts (Lindsay et al., 1996). By observing the characteristics of the sarcocysts it was determined that Sarcocystis dasypi was present in 21 of the armadillos and Sarcocystis diminuta was present in 5 of the armadillos, with 3 armadillos showing signs of a mixed infection (Lindsay et al., 1996). Another study conducted in Florida showed that 39 of 63 armadillos contained Sarcocystis sarcocysts (DeLucia et al., 2002). These studies showed that infection of armadillos with Sarcocystis species is common in North America.

Work establishing that S. neurona sarcocysts were present in armadillos using tongues from road-killed armadillos (Tanhauser et al., 2001). DNA was extracted and characterized using PCR amplification followed by restriction fragment length polymorphism analysis and nucleotide sequencing. A 254-nucleotide sequence was determined to be identical to S. neurona for each sample (Tanhauser et al., 2001). In addition to the DNA research, sera was examined from 19 wild caught armadillos and 17 captive raised armadillos using a western-blot technique developed to detect anti-S. neurona antibodies. All of the wild caught armadillos and 1 of the captive raised armadillos had antibodies for S. neurona suggesting that armadillos are naturally infected with S. neurona (Tanhauser et al., 2001).
The armadillo was established as an intermediate host for *S. neurona* when sarcocyst infected tissue samples were collected from an assortment of automobile-killed, live trapped, and killed armadillos which were fed to wild caught and laboratory raised naïve opossums (Cheadle et al., 2001b). Sarcocysts from infected tissues were also analyzed using DNA extraction and amplification techniques and found to contain evidence supporting they were infected with *S. neurona* (Cheadle et al., 2001b; Tanhauser et al., 2001). Sporocysts were collected from opossums using gastrointestinal scraping techniques. These sporocysts were then orally inoculated into an immune-competent foal. The foal developed clinical signs consistent with EPM and both CSF and serum tested positive for *S. neurona* antibodies (Cheadle et al., 2001b). In a subsequent study, sporocysts collected from the previous opossums were used to infect gamma-interferon KO mice (Cheadle et al., 2002). This resulted in KO mice developing encephalitis and the *S. neurona* merozoites were found in the brains of mice when analyzed using immunohistochemical staining techniques. These studies established that armadillos are naturally infected with *S. neurona* and serve as a viable intermediate host (Cheadle et al., 2002).

### 2.8.4 Sea Otters as Intermediate Hosts

Sea otters (*Enhydra lutris*) are marine mammals that are found along the pacific coast of the United States and Canada as well as the eastern coast of Japan (Burton and Burton, 2002). They usually remain within a ½ mile of the shoreline and are often located around the kelp forests off the coast of California. Their diet consists mainly of shelled invertebrates and occasionally small fish and octopus (Burton and Burton, 2002).
The first report of *Sarcocystis* infection in a species of sea otters, *Lutra lutra*, was reported from captive sea otter raised in Norway (Wahlstrom et al., 1999). Sarcocysts were analyzed using light and electron microscopy and found to contain few metrocytes and numerous bradyzoites (Wahlstrom et al., 1999). A captive sea otter that developed CNS signs was diagnosed with a *S. neurona*-like organism based on antibodies detected in the CSF by Western blot analysis (Rosonke et al., 1999). Immunohistochemical staining of the spinal cord and brain of this sea otter indicated a *S. neurona*-like organism was present (Rosonke et al., 1999). *S. neurona* was isolated from a different sea otter suffering from CNS disease using immunohistochemical and PCR amplification techniques (Lindsay et al., 2000). Interferon gamma KO mice that were inoculated with merozoites collected from the affected otter developed *S. neurona* associated encephalitis and had antibodies to *S. neurona* in serum (Lindsay et al., 2000). *S. neurona* was isolated from free-ranging sea otter that developed meningoencephalitis (Miller et al., 2001). When the isolate obtained from this sea otter (SO SN1) was tested against a known *S. neurona* isolate it was indistinguishable based on parasite morphology, antigenic reactivity and molecular characterization (Miller et al., 2001).

*S. neurona* was characterized from sarcocyst infected muscle tissues from two naturally infected sea otters using DNA extraction and PCR amplification (Dubey et al., 2001e). The structures of these sarcocysts were similar to those seen in *S. neurona* infected cats. Sarcocyst infected muscle tissues were fed to laboratory raised opossums (Dubey et al., 2001e). These opossums shed sporocysts that were then inoculated into KO mice. *S. neurona* was identified in the tissues of these mice using
immunohistochemistry. This study supported that sea otters are naturally infected with *S. neurona* and can act as an intermediate host for the parasite (Dubey et al., 2001d).

The first report of localized epizootic in marine wildlife caused by apicomplexan parasites was due to *S. neurona* infections in sea otters (Miller et al.). 40 sick and dead sea otters were found along an 18 km stretch of Californian coastline. Of the sea otters tested, 94% of them tested positive for *S. neurona* infection based on PCR amplification and sequencing and 100% of those tested had an elevated titer to *S. neurona* IgG and IgM (Miller et al.). The reason there was such a dramatic occurrence of disease is unknown but may be related to marine biotoxin domoic acid that may have enhanced susceptibility to disease or it could have been due to an abundant amount of rain runoff due to a large storm in the area (Miller et al.).

The ability of *S. neurona* to infect marine mammals was unexpected since it does not seem to play a role in the development of the life cycle (Miller et al.; Dubey et al., 2001e). How the sea otter become infected with *S. neurona* is unknown. It has been theorized that incidental exposure to sporocysts shed by opossums in infected rain runoff may play a role, however an unknown paratenic host or unknown natural predator of sea otters that can shed sporocysts has not been ruled out (Dubey et al., 2001d). It is possible that the sea otter is an incidental intermediate host of *S. neurona* and as such is more severely affected by the parasite when compared to the other intermediate hosts. For most of the intermediate hosts to show clinical signs of illness they are first found to be immunoincompetent and therefore more susceptible to disease by *Sarcocystis* species. This does not seem to be the case for the sea otter which can become severely infected by *S. neurona* without any complicating factors.
2.8.5 Raccoons as Intermediate Hosts

Raccoons (*Procyon lotor*) are one of the most readily recognized mammals in North America (Burton and Burton, 2002). The distribution of raccoons ranges from Canada to Central America. They are mostly nocturnal, solitary animals that prefer woods and brush areas but have adapted to open country as their habitats are slowly destroyed. In cold northern climates, raccoons semi-hibernate during exceptionally cold times, in contrast southern raccoons remain active year round (Burton and Burton, 2002). Like cats, raccoons are primarily considered carnivores and therefore they also don’t fit the normal predator-prey cycle associated with *Sarcocystis* species. The first report of *Sarcocystis* infection in raccoons was due to a survey conducted in Michigan looking at prevalence of sarcocysts in feral and domestic animals slaughtered in the area (Seneviratna et al., 1975).

The raccoon has been reported to shed sporocysts in the wild as well as in the laboratory setting and has been identified as the definitive host for *S. cruzi* and *S. miescheriana* (Fayer et al., 1976; Prestwood et al., 1980; Adams et al., 1981; Dubey et al., 1989). Sarcocysts of *Sarcocystis* sp were found in 26 of 52 raccoons that were surveyed in Ohio, Pennsylvania, Florida, and Maryland (Kirkpatrick et al., 1987). This study showed a significant amount of sarcocysts seen in the tongue, diaphragm, masseter muscle and esophagus of infected raccoons. Skeletal and heart muscle obtained from 66 of 100 raccoons studied in Illinois reveal positive sarcocysts of *Sarcocystis kirkpatricki* (Snyder et al., 1990). Prior to a report of a raccoon with fatal necrotizing encephalitis due to a *Sarcocystis*-like protozoan infection, only *T. gondii* and *Neospora caninum* were known to cause encephalomyelitis in carnivores due to a coccida infection (Dubey et al.,
1990). Subsequent infection with canine distemper virus has been known to increase the likelihood of raccoons obtaining fatal encephalitis due to *Sarcocystis* infection (Stoffregen and Dubey, 1991; Thulin et al., 1992).

The same study that established that *S. neurona* was found in the CNS of skunks was also the first to demonstrate that *S. neurona* organisms could be isolated from the CNS of a raccoon (Dubey and Hamir, 2000). A possible association of myocarditis in conjunction with *S. neurona* sarcocyst infection was demonstrated in 2 raccoons (Hamir and Dubey, 2001). In this study *S. neurona* was demonstrated in the CNS through immunohistochemistry and both raccoons reacted positively with *S. neurona* antibodies. These raccoons were found to be immunocompetent and demonstrated no clinical signs of encephalitis. However, a finding of interest in this study was that both raccoons were found in close proximity to horse facilities which suggested that equines in these geographic locations were at higher risk for developing EPM (Hamir and Dubey, 2001).

The raccoon was established as a natural intermediate host for *S. neurona* when sarcocyst infected tongues from naturally infected raccoons were fed to laboratory raised opossums (Dubey et al., 2001d). These opossums then shed sporocysts that were collected using a GI scraping technique and orally inoculated into interferon-gamma KO mice. KO mice developed neurologic signs and *S. neurona* was identified in tissues using immunohistochemical staining (Dubey et al., 2001d). Raccoons that were fed sporocysts from infected opossums, developed *S. neurona*-associated encephalitis, and schizonts and merozoites were seen in encephalitic lesions. In an attempt to prove that the raccoons could not act as a definitive host for *S. neurona* sarcocysts, the infected
tissues from the tongues were also fed to laboratory raised raccoons and no sporocysts were detected in the feces of these raccoons (Dubey et al., 2001d).

Seroprevalence for *S. neurona* antibodies in horses from United States is estimated to be between 33-60% (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Rossano et al., 2001). Seroprevalences found in raccoons have proven to be similar to those reported for horses (Lindsay et al., 2001). Raccoons from Florida, New Jersey, Pennsylvania, and Massachusetts were examined for agglutinating antibodies to *S. neurona* using a *S. neurona* agglutination test (SAT) (Lindsay et al., 2001). 58 of 99 (58.6%) of raccoons tested positive for *S. neurona* antibodies. A seroprevalence study for *S. neurona* antibodies found in raccoons from metropolitan areas indicated a much higher prevalence at 92.3% (Hancock et al., 2004). The prevalence studies and the establishment of raccoons as an intermediate host for *S. neurona* helps explain the large geographic distribution associated with EPM.

### 2.8.6 Importance of Understanding the Life Cycle of *S. neurona*

By understanding the life cycle of *S. neurona* we are better able to understand how the disease is transmitted throughout the Americas and how the disease is distributed within the intermediate and aberrant hosts (Blythe et al., 1997; Dubey et al., 2000b; Elitsur et al., 2007). Elitsur et al. has done the only study to demonstrate early stages of infection of *S. neurona* distributed in the horse (Elitsur et al., 2007). Currently the gold standard for diagnosing EPM in horses is post mortem examination, however the accepted antemortem diagnostic is Western blot (Immunoblot) analysis for the detection of *S. neurona* antibodies in the serum and cerebrospinal fluid (CSF) of horses (Granstrom
et al., 1993). The Western Blot technique utilizes cultivation of *S. neurona* merozoites, probing them with serum collected from suspected horses and using primary and secondary antibodies to identify specific proteins (22.5, 13, and 10.5kd) that correspond to proteins found in EPM cases and *S. neurona* inoculated horses (Granstrom et al., 1993). There are some draw backs to this diagnostic testing. While this test allows for the detection of antibodies to *S. neurona* within a given animal it does not represent concurrent disease within that animal, it is also very costly to maintain the merozoites within the laboratory. Often the merozoites are injected into an intermediate host which muscle tissue from the infected host is then given to the definitive host for the harvesting of additional sporocysts to be used in further experimentation. This is also costly and timely as it takes approximately 75 days for the intermediate hosts to develop sarcocysts (Dubey et al., 1989).

The first study to demonstrate that culture derived merozoites could be directly infective for the intermediate host utilized feline and equine-derived merozoites that were experimentally inoculated into cats (Butcher et al., 2002). While the feline-derived merozoites were able to infect the cats the equine-derived merozoites were not able to cause infection in cats thus suggesting a possible biological difference between these merozoites (Butcher et al., 2002). Serum from the infected cats was then tested using the immunoblot analysis for the detection of *S. neurona* antibodies and was found to be positive reaction in all infected cats (Butcher et al., 2002). However, there was no distinct serological difference between cats that were infected with the cat strain of *S. neurona* parasites compared to those infected with the equine derived strains of *S. neurona* parasites (Butcher et al., 2002).
Further understanding of how merozoites are transmitted within the intermediate hosts is needed. The effects of the different strains of *S. neurona* and their ability to cause infection within an intermediate host will help us to understand the development of this parasite and possibly lead to increased diagnostics and possible prevention of the disease.
2.9. REFERENCES


Brocke, R., 1970. The winter ecology and bioenergetics of the opossum, Didelphis marsupialis, as distributional factors in Michigan. Michigan State University.


Howe, D.K., Gaji, R.Y., Mroz-Barrett, M., Gubbels, M.J., Striepen, B., Stamper, S., 2005. Sarcocystis neurona merozoites express a family of immunogenic surface antigens that are orthologues of the Toxoplasma gondii surface antigens (SAGs) and SAG-related sequences. Infect Immun 73, 1023-1033.


Krause, W.J., Krause, W.A., 2006. The Opossum: Its Amazing Story. Department of Pathology and Anatomical Sciences, School of Medicine, University of Missouri, Columbia, Missouri.


Sarcocystis neurona in raccoons, Procyon lotor, from the United States. Vet Parasitol 100, 131-134.


Miller, M.A., Conrad, P.A., Harris, M., Hatfield, B., Langlois, G., Jessup, D.A., Magargal, S.L., Packham, A.E., Toy-Choutka, S., Melli, A.C., Murray, M.A.,


 CHAPTER 3 : EXPERIMENTAL INOCULATION OF RACCOONS (*PROCYON LOTOR*) WITH DIFFERENT STRAINS OF *SARCOCYSTIS NEURONA* MEROZOITES

3.1. INTRODUCTION

The apicomplexan parasite *Sarcocystis neurona* is one of the causative agents of equine protozoal myeloencephalitis (EPM), a severe neurological disease in horses (Dubey et al., 2001b). The mode of transmission of *S. neurona* is a fecal-oral transmission from the definitive host (*Didelphis virginiana*), which excretes sporocysts and oocysts in their feces (Dubey and Lindsay, 1998). The aberrant host, the horse (*Equus caballus*), and the normal intermediate hosts, which include the raccoon (*Procyon lotor*) (Dubey et al., 2001c), the sea otter (*Enhydra lutris*) (Dubey et al., 2001d), the nine banded armadillo, (*Dasypus novemcinctus*) (Cheadle et al., 2001a; Tanhauser et al., 2001), the domestic cat (*Felis domesticus*) (Dubey et al., 2000), and the striped skunks (*Mephitis mephitis*) (Cheadle et al., 2001b), become infected by ingesting food or water that is contaminated with sporocysts (Dubey et al., 2000).

Our hypothesis was that laboratory grown merozoites that were inoculated by 3 alternate methods (intravenous, subcutaneous and intramuscular) would result in sarcocyst development in raccoons. The basis of this study was to ascertain if there are any biological differences between isolates by evaluating their ability to encyst in raccoons and later form sporocysts in opossums.
3.2. MATERIALS AND METHODS

3.2.1 Experimentally-infected Raccoons

Raccoons (n=6) were acquired at 10 weeks of age from a commercial fur dealer (Ruby Fur Farm, New Sharon, Iowa) where they were fed a previously described diet (Dubey et al., 2001c). Raccoons were allowed to acclimatize to their environment for 7 days prior to inoculation. Raccoons 1 through 4 were experimentally inoculated with $1 \times 10^7$ merozoites of *S. neurona* divided equally by three routes of infection: intravenous (IV), intramuscular (IM), and subcutaneous (SQ). Merozoites were propagated in equine dermal cells for in vivo inoculation.

Each raccoon was inoculated with merozoites from one of four different *S. neurona* isolates: raccoon 1 was inoculated with the sea otter derived Sn-OT-1 isolate (Dubey et al., 2001d); raccoon 2 was inoculated with the raccoon derived Sn-37-R isolate (Dubey et al., 2001c); raccoon 3 was inoculated with the equine derived Sn-UCD1 isolate (Marsh et al., 1996); and raccoon 4 was inoculated with the cat derived Sn-Mucat2 isolate (Turay et al., 2002). Positive control raccoons 5 and 6 were orally inoculated with $3 \times 10^7$ *S. neurona* sporocysts of the Sn-37-R isolate, which were allowed to saturate a small portion of dry dog food and fed to raccoons after a 12h period of fasting as previously described (Table 1) (Stanek et al., 2002).

Raccoons were housed separately and were maintained on a diet of dry dog food and water. Raccoons were observed daily for signs of neurologic disease (Stanek et al., 2002). Blood was collected on the day of inoculation and again on the day of termination. Raccoons were humanely terminated by CO$_2$ asphyxiation 75 days after inoculation.
### 3.2.2 Histology and Immunohistochemistry

During necropsy, sections from the tongue, masseter, temporalis, quadriceps, liver, back muscle, heart, diaphragm, lungs, brain, and spinal cord were fixed in 10% neutral buffered formalin. These tissues were then embedded in paraffin and analyzed for the detection of tissue cysts using hematoxylin and eosin staining techniques (Table 2).

Immunohistochemistry (IHC) was performed on tissue sections from raccoon 1, (Sn-OT-1); raccoon 2, (Sn-37-R); and raccoon 5, which was orally inoculated with Sn-37-R. Control tissue included an experimentally infected raccoon with encephalitis (Stanek et al., 2002) and a *S. neurona* (Sn-37-R) infected interferon gamma KO mouse with encephalitis (Dubey et al., 2001a). Rabbit polyclonal anti-sera raised against *S. neurona* were used as previously described (Butcher et al., 2001). In addition, a polyclonal monospecific antibody to *S. neurona* merozoite surface antigen (SnSAG 4) (Howe et al., 2005), was used at 1:1000 using the same protocol (Table 3). The staining utilized the Dako Envision Plus System. Normal rabbit serum was used as a negative control.

### 3.2.3 Feeding Trials of Opossums

This study included 8 laboratory raised opossums, weaned from a pregnant wild caught opossum as previously described (Dubey et al., 2000). Opossums were housed individually and maintained on dry dog food and water. Opossums were examined for 4 days prior to the start of the study for the shedding of sporocysts using a sugar flotation as previously described (Dubey et al., 1989). The opossums were approximately 8
months of age when they first received raccoon tissues. Each opossum received an individual amount of minced muscle tissue daily from an infected raccoon (Table 1). Fecal samples were collected daily and analyzed by sugar floatation for sporocyst shedding.

Opossums were terminated when sporocysts were detected in the feces. If no sporocysts were detected, opossums were terminated at 33 days or 44 days after inoculation (Table 2). Opossums were euthanized by CO₂ asphyxiation and Euthasol (Virbac Corporation, Fort Worth, TX). After euthanasia, the small intestine from all opossums were removed and tied at the ileac and pyloric ends. Intestines from opossums 5, 6, 7, and 8 were shipped on ice to the USDA laboratory (Beltsville, MD) where they were processed for detection of sporocysts (Dubey et al., 2000). The intestines from opossums 1 (Sn-OT-1), 2 (Sn-37-R), 3 (Sn-UCD), 4 (Sn-Mucat 2) were processed at OSU for the detection of sporocysts in a similar manner (Dubey et al., 2000).

3.2.4 Serological Examination of S. neurona antibodies

Sera collected from raccoons on the day of inoculation and the day of termination were analyzed using a modification of the original Immunoblot assays (Granstrom et al., 1993). S. neurona merozoites from the Sn-37-R isolate and the Sn-OT-1 isolate were cultured in equine dermal cells for antigen. Merozoites were harvested from culture and centrifuged at 300 g at 10°C for 10 min. The merozoites were washed twice with PBS and counted. The final pellet was resuspended in 200-400µl of sterile water and underwent 2 rapid freeze thaw cycles to lyse the parasite. Nitrocellulose membranes were prepared for Immunoblot analysis following manufacturers recommended protocol
(Invitrogen, Carlsbad, California). NuPAGE LDS Sample Buffer was added in equal volume to 3.6 x 10^6 merozoites in solution. Samples were heated at 90°C for 5 min, loaded onto NuPAGE 4-12% Bis-Tris gels and electrophoresed using NuPAGE MES SDS Running buffer (Invitrogen) at 100V constant voltage. Proteins were transferred to nitrocellulose membrane using transfer buffer (50mM Tris, 380mM Glycine, 0.1% SDS, 20% methanol).

The membranes were blocked using 5% dry milk in 0.01% TBS-Tween solution. Membranes were probed with individual raccoon serum (1:50), for 1 hour at room temperature and washed 3 times in TBS-Tween. Rabbit anti-raccoon affinity purified IgG conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody. Membranes were developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate) (nitro blue tetrazolium) membrane phosphatase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

3.3. **RESULTS**

3.3.1 **Sarcocyst Detection in Tissues Following Inoculation of S. neurona**

There were no clinical signs of illness neither in the raccoons that ingested sporocyst nor in the raccoons that were injected with merozoites. Sarcocysts were seen in histological sections from raccoon 1, which was inoculated with Sn-OT-1 merozoites, and raccoon 2, which was inoculated with Sn-37-R merozoites. Both positive control raccoons developed tissue cysts as well. Sarcocysts were detected in tissue sections from the tongue, masseter muscles, temporalis muscles, quadriceps muscles, deltid muscles, and the diaphragm (Table 4). When analyzed under light microscope, there were no
detectable cysts seen in tissues collected from raccoons 3 and 4, which were inoculated with Sn-UCD1 and Sn-Mucat2 respectively.

### 3.3.2 Immunohistochemical Staining for the Detection of SnSAG4

Immunohistochemistry performed on the tissue sections from a raccoon that developed encephalitis (Stanek et al., 2002) and a *S. neurona* infected gamma-interferon KO mouse (Dubey, 2001) stained positive for parasite antigen when using the rabbit polyclonal antiserum against *S. neurona*. These tissue sections also showed positive staining of merozoites when the monospecific polyclonal antiserum against SnSAG4 was used. In contrast, the Sn-OT-1 sarcocysts and the Sn-37-R sarcocysts did not show positive staining with either antiserum (Table 3). There was no staining seen of any of the tissue samples when normal rabbit serum was used.

### 3.3.3 Opossum Feed Trials

The feeding trials and sporocyst detection are summarized in Table 2. Tissues containing cysts from raccoon 1 (Sn-OT-1 inoculated) were fed to opossum 1, and tissues containing cysts from raccoon 2 (Sn-37-R inoculated) were fed to opossum 2. There were no sporocysts detected in feces from either opossum when analyzed using fecal flotation. However gastrointestinal scrapings from opossum 1 (Sn-OT-1 inoculated) and opossum 2 (Sn-37-R inoculated) each revealed a small number of sporocysts. Tissues from raccoon 3 (Sn-UCD1 inoculated) and raccoon 4 (Sn-Mucat2 inoculated) were each fed respectively to opossum 3 and opossum 4. Both fecal sampling and gastrointestinal scrapings from these opossums were negative for sporocysts.
The two positive control raccoons, which were orally inoculated with Sn-37-R and developed tissue cysts, were each fed to two opossums; tissues from raccoon 5 were fed to opossum 5 and 6, whereas tissues from raccoon 6 were fed to opossums 7 and 8. Seventeen days after the initiation of feeding trials, sporocysts were detected in the feces of opossum 5. Sporocysts were detected in the feces of opossum 6 after 21 days. Sporocysts were detected in the feces of opossum 7 sixteen days after onset of the feeding trial; however, no sporocysts were detected in the feces of opossum 8 up to 33 days after the initiation of the feeding trials. Gastrointestinal scrapings for opossums 5, 6, 7, and 8 were all positive for sporocysts. However, there were less sporocysts detected in the scraping from opossum 8 when compared to the number detected in the other three opossums, whose amounts were comparable.

3.3.4 Immunoblot Analysis

Pre-inoculation sera from all raccoons showed no reactivity to the *S. neurona* merozoites in western blot analysis. Antibodies to *S. neurona* were detected in all raccoons 75 days post inoculation when tested against merozoite antigens Sn-37-R and Sn-OT-1 isolates. There were distinguishable differences in sera immunoreactivity to the different antigens (Fig. 1). Specifically, the approximately 26kDa bands showed the most significant differences between the immunoblots. Raccoon 1 (Sn-OT-1 inoculated) reacted stronger to the ~26 kDa antigens using Sn-OT-1 merozoite antigen as compared to the antigen from the Sn-37-R merozoites. In contrast, raccoon 2 (Sn-37-R inoculated) reacted stronger to the ~26 kDa antigens using Sn-37-R merozoite antigens as compared to antigen from the Sn-OT-1 merozoites. Little to no difference in reactivity to the
approximately 13kDa band was observed amongst the raccoon sera when tested against each parasite isolate.

### 3.4. DISCUSSION

It was determined that culture-derived *S. neurona* merozoites from the Sn-OT-1 isolate and the Sn-37-R isolates were capable of encysting in raccoons after non-oral inoculation. Furthermore, these tissue cysts were infectious when they were ingested by the definitive hosts, as the parasites were able to undergo sexual reproduction and form oocysts and sporocysts. This was based upon detection of the bradyzoites in the tissues from the two raccoons receiving culture-derived merozoites by parenteral inoculation. Sporocysts were then detected in the intestines of the opossums that ingested the infected muscle tissues of these raccoons.

*S. neurona* merozoites derived from the naturally occurring raccoon strain Sn-37-R (Dubey et al., 2001c) successfully developed infectious tissue cysts when experimentally inoculated into a raccoon. There was a significant decrease in the number of sporocysts detected in the intestinal scrapings when compared to opossums that ingested tissue from an orally inoculated, control raccoon. The low number of sarcocysts ingested likely contributed to this observation, since only gametogony and no asexual proliferation occurs in the definitive host (Dubey et al., 1989). Since the number of merozoites was divided equally between the three non-oral inoculation routes, intravenous, intramuscular, and subcutaneous, the number of merozoites from each route that were able to eventually encyst is unknown. Further studies are needed to determine which method of inoculation and parasite concentration most effectively causes parasite
infection and sarcocyst formation in the intermediate host. Optimization of this infection model may enable investigators to artificially modulate parasite infection with culture-derived merozoites in the intermediate host.

This study confirms earlier findings that unlike other species of *Sarcocystis*, *S. neurona* merozoites can be infectious for the intermediate host and can form mature sarcocysts. Dubey (Dubey et al., 2002) reported that of the 3 cats inoculated with 1 million or more merozoites of an *S. neurona* opossum isolate developed mature sarcocysts that were infective for the definitive host. Butcher (Butcher et al., 2002) found sarcocysts in muscles of 2 cats inoculated with merozoites of the Sn-Mucat2 but not in 2 cats inoculated with the Sn-UCD1 isolate; sarcocysts from one of the cats inoculated with the Sn-Mucat2 isolate were infective for the opossum. In this study, sarcocysts were found in the muscle of raccoons inoculated with SN-37-R and Sn-OT-1 but not with the Sn-Mucat2 and the Sn-UCD1. Whether these differences are related to the origin of the isolate or the poor infectivity of the merozoite stage needs investigation. Nevertheless, formation of mature sarcocysts in a laboratory animal after inoculation with merozoites allows for completion of the life cycle under experimental conditions.

The immunoblot analysis showed the antigenic difference between the *S. neurona* isolates. There were distinct differences between the banding patterns for the antibodies of the Sn-OT-1 isolate and the Sn-37-R isolate when evaluated against the different antigen preparations. If the 2 isolates were identical similar reactivity to the different antigens should occur from both sets of antibodies. Further experiments where the Sn-37-R and Sn-OT-1 isolates were inoculated into sea otters and sera experimentally tested
would further help evaluate the infectivity of the strains and evaluate antigenic differences.

The tissue cyst stages of *S. neurona* (Sn-37-R isolate and Sn-OT-1 isolate) did not react with either of the anti-*S. neurona* antisera or with the monospecific polyclonal antiserum against SnSAG4. This is not surprising as others have shown that antibodies produced against a specific stage in a life cycle might have little to no reaction to antigens from a different stage of the life cycle ((Dubey and Lindsay, 2003). The absence of sarcocyst staining by antiserum against SnSAG4 suggests that expression of this surface antigen might be stage specific, as observed previously for many of the homologous surface antigens in *Toxoplasma gondii* (Lekutis et al., 2001). Further studies involving testing merozoites reactivity from a naturally infected sea otter, with documented parasite-associated encephalitis, may reveal further similarities or differences between the two isolates in a different stage of the life cycle.

It is important to note that despite the apparent absence of sporocysts in the feces of opossum 8, there were a significant number of sporocysts harvested from GI scrapings. Also, opossum 1 and opossum 2 showed no signs of sporocyst shedding during fecal analysis, although sporocysts were detected in GI scrapings. This suggests that scraping is a more sensitive method for detecting sporocysts and therefore is a better indicator of opossum infections. Fecal flotation may be unreliable if used as the only method of detecting sporocysts.

This is the first study to demonstrate that culture-derived *S. neurona* merozoites (Sn-37-R and Sn-OT-1) have the ability to encyst after non-oral inoculation into raccoons. This experiment indicates that there are biological and antigenic differences
between *S. neurona* isolates. Further study is necessary to determine the level of divergence between different isolates which may contribute to their pathogenicity. This may determine their usefulness in an experimental model to improve diagnostics and develop prevention programs.
3.5. FIGURES AND TABLES

Figure 1: Westernblots were conducted using primary antibodies (a) Sn-37-R (b) Sn-OT-1 and secondary antibody rabbit anti-raccoon IgG. Raccoon serum was loaded as follows: Lane 1: Sn-OT-1, Lane 2: Sn-37-R, Lane 3: Sn-UCD, Lane 4: Sn-Mucat-2, Lanes 5&6: Sn-37-R (orally inoculated)
Figure 2: (a) IHC tests were run on Raccoon diaphragm tissue using the TRS method for detecting reactions to the SAG4 antigen. Arrow points out bradyzoites found in the muscle tissue. Lack of staining indicates that no SAG4 antigens were present. (b) IHC tests were run on control mouse tissue using the PK method for detecting reactions to the SAG4 antigen. Arrow points out merozoites in muscle tissue. Merozoites stained positive for the presence of SAG4 antigen.
**Table 1:** Experimental inoculations of raccoon, serological analysis, tissue analysis

<table>
<thead>
<tr>
<th>Raccoon</th>
<th>Inoculum</th>
<th>Mode of Inoculation</th>
<th>Parasite #</th>
<th>Seroconversion*</th>
<th>Tissue cysts†</th>
<th>Opossum Feeding</th>
<th>Sporocysts‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sn-OT-1</td>
<td>IV, SQ, IM</td>
<td>1x10⁷</td>
<td>Positive</td>
<td>Positive</td>
<td>Opossum 1</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Sn-37-R</td>
<td>IV, SQ, IM</td>
<td>1x10⁷</td>
<td>Positive</td>
<td>Positive</td>
<td>Opossum 2</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>Sn-UCD1</td>
<td>IV, SQ, IM</td>
<td>1x10⁷</td>
<td>Positive</td>
<td>Negative</td>
<td>Opossum 3</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Sn-Mucat-2</td>
<td>IV, SQ, IM</td>
<td>1x10⁷</td>
<td>Positive</td>
<td>Negative</td>
<td>Opossum 4</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Sn-37-R</td>
<td>Oral</td>
<td>3x10⁷</td>
<td>Positive</td>
<td>Positive</td>
<td>Opossum 5, 6</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Sn-37-R</td>
<td>Oral</td>
<td>3x10⁷</td>
<td>Positive</td>
<td>Positive</td>
<td>Opossum 7, 8</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*Amount of parasite used to inoculate raccoon

* Serum analysis was based on results from Western Blots using Sn-OT-1 and Sn-37-R as antigen

† Analysis based on hemotoxylin and eosin stained tissue section

‡ Positive: Tissue sarcocysts discovered by microscopic analysis; Negative no tissue sarcocysts

§ Negative: No microscopic analysis of sporocysts; Positive Sporocysts were detected
**Table 2:** Detection of sporocysts via fecal analysis and gastrointestinal scrapings

<table>
<thead>
<tr>
<th>Opossum</th>
<th>Days Post Initiation of feeding</th>
<th>Fecal*</th>
<th>Gastrointestinal scraping†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>33</td>
<td>Negative</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Microscopic analysis of sporocysts via fecal floatation
† Microscopic analysis of sporocysts harvested from GI scraping
<table>
<thead>
<tr>
<th></th>
<th>R81</th>
<th>SAG 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Mouse ‡</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Encephalitis Raccoon §</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Raccoon 1 ¶</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Raccoon 2 #</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Anti-*-*S. neurona* antibody  
† Polyclonal monospecific antibody to *S. neurona*  
‡ *S. neurona* infected interferon gamma knockout mouse with encephalitis  
§ *S. neurona* infected raccoon with encephalitis (Stanek et. Al)  
¶ Experimentally infected with *Sn-OT-1* merozoites  
# Experimentally infected with *Sn-37-R* merozoites
Table 4: Results of hemotoxylin and eoson stained tissue section

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Raccoon 1</th>
<th>Raccoon 2</th>
<th>Raccoon 3</th>
<th>Raccoon 4</th>
<th>Raccoon 5</th>
<th>Raccoon 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tongue</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Masseter</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Temporalis</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Quadriceps</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Liver</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Deltoid muscle</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Heart</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Lung</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*Positive: tissue sarcocysts detected by microscopic analysis

†Negative: No tissue sarcocysts detected by microscopic analysis
3.6. REFERENCES


opossums (Didelphis virginiana), and experimental induction of neurologic disease in raccoons. Vet Parasitol 100, 117-129.


Howe, D.K., Gaji, R.Y., Mroz-Barrett, M., Gubbels, M.J., Striepen, B., Stamper, S., 2005. Sarcocystis neurona merozoites express a family of immunogenic surface antigens that are orthologues of the Toxoplasma gondii surface antigens (SAGs) and SAG-related sequences. Infect Immun 73, 1023-1033.


BIBLIOGRAPHY


Brocke, R., 1970. The winter ecology and bioenergetics of the opossum, Didelphis marsupialis, as distributional factors in Michigan. Michigan State University.


Howe, D.K., Gaji, R.Y., Mroz-Barrett, M., Gubbels, M.J., Striepen, B., Stamper, S., 2005. Sarcocystis neurona merozoites express a family of immunogenic surface antigens that are orthologues of the Toxoplasma gondii surface antigens (SAGs) and SAG-related sequences. Infect Immun 73, 1023-1033.


Krause, W.J., Krause, W.A., 2006. The Opossum: Its Amazing Story. Department of Pathology and Anatomical Sciences, School of Medicine, University of Missouri, Columbia, Missouri.


Sarcocystis neurona in raccoons, Procyon lotor, from the United States. Vet Parasitol 100, 131-134.


Miller, M.A., Conrad, P.A., Harris, M., Hatfield, B., Langlois, G., Jessup, D.A., Magargal, S.L., Packham, A.E., Toy-Choutka, S., Melli, A.C., Murray, M.A.,


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