NATURALLY OCCURRING *Sarcocystis* INFECTION IN DOMESTIC CATS (*Felis catus*)

By

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NATURALLY-OCCURRING *Sarcocystis* INFECTION IN DOMESTIC CATS

(*Felis catus*)

By

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Equine protozoal myeloencephalitis is an important neurological disease of horses in the United States. Consequently, there is an active research effort to identify hosts associated with the primary causative agent, *Sarcocystis neurona*. The purpose of this study was to determine whether the domestic cat (*Felis catus*) is a natural host for *S. neurona*. Muscle sections from 50 primarily free-roaming domestic cats were examined for the presence of sarcocysts. Sera from cats in this group and another group of 50 free-roaming cats were evaluated for the presence of *S. neurona* antibody. Sarcocysts were found in 5 of 50 cats (10%), and *S. neurona* antibody was found in 5 of 100 cats (5%) (one of which was also infected with sarcocysts). Morphological, molecular (including ribosomal RNA genes), and biological characterization of these sarcocysts showed that they were not *S. neurona* or *S. neurona*-like. Sarcocysts found in the cats were identified morphologically as *Sarcocystis felis*, a common parasite of wild felids. The life cycle of *S. felis* is not known; before this study, no molecular marker for

S. felis existed. Although cats were found to be infected with *S. felis* sarcocysts, serological data provided evidence of possible infection with *S. neurona* as well. Further work is needed to determine the role of the domestic cat in the life cycle of *S. neurona*.

CHAPTER 1
INTRODUCTION

Sarcocystis spp. are common parasites with a wide range of hosts. Their life cycles are unusual in that they are perpetuated by using two hosts: a definitive host that eats sarcocyst-infected muscle, then passes infective sporocysts; and an intermediate host that ingests these sporocysts and forms muscle sarcocysts as a result (Dubey et al., 1989). A comprehensive cataloging of *Sarcocystis* spp. (Odening, 1998) indicates 189 species of *Sarcocystis* have been identified to date; however, both definitive and intermediate hosts are known for only 46% of these species. That report lists domestic cats (*Felis catus*) as definitive hosts for 15 species, and as an intermediate host for only one species, *Sarcocystis felis*.

The domestic cat's role as an intermediate host for another species, *Sarcocystis neurona*, has implications for the equine industry, resulting in several recent publications and prompting the writing of this thesis as well. *Sarcocystis neurona* is the primary causative agent of equine protozoal myeloencephalitis (EPM), an important neurological disease of horses (Dubey et al., 1991, reviewed in MacKay et al., 2000). Currently, effective preventatives for *S. neurona* infection (and thus EPM) in horses are neither widely available nor widely used, so there is considerable interest in defining and controlling the natural hosts of this parasite. Opossums (*Didelphis virginiana, Didelphis albiventris*) are definitive hosts for *S. neurona* (Fenger et al., 1995, Dubey et al., 2001e); nine-banded armadillos (*Dasypus novemcinctus)* (Cheadle et al., 2001a, Tanhauser et al., 2001) and raccoons (*Procyon lotor*) (Dubey et al., 2001a) have been implicated as natural

intermediate hosts. The striped skunk (*Mephitis mephitis*) has also been identified as an intermediate host, but only under laboratory conditions (Cheadle et al., 2001b).

The domestic cat may also be an intermediate host for *S. neurona.* In 2000, Dubey and co-workers (2000) reported the first experimental completion of the life cycle of *S. neurona*. Sarcocyst formation was induced in four laboratory-raised cats that had been dosed with large numbers of *S. neurona* sporocysts obtained from naturally-infected opossums. Three of these cats were immunosuppressed by corticosteroid administration. Sarcocysts that formed in cat muscle were infective for two opossums. Sporocysts produced by the infected opossums in turn caused *S. neurona*-associated encephalitis in immune-deficient, interferon-gamma knockout mice. Molecular analysis of merozoites recovered from the brain of one cat was consistent with *S. neurona*. Additional studies have further defined *S. neurona* infection in cats (Stanek et al., 2001, Turay et al., 2002, Butcher et al., 2002, Dubey et al., 2002, Rossano et al., 2002).

Before the discovery that the cat is able to function as an intermediate host for *S. neurona*, the only other *Sarcocystis* spp. for which the cat was known to be an intermediate host was *Sarcocystis felis* (Dubey et al., 1992), named for the organism that formed sarcocysts in the muscle of bobcats (*Felis rufus*). In that report, it was noted that, based on ultrastructural morphology, these sarcocysts were identical to those found in the domestic cat (*Felis catus*) and in cougars and panthers (*Felis concolor*). Later, *S. felis* was formally identified in cheetahs (*Acinonyx jubatus*) (Briggs et al., 1993) and lions (*Panthera leo*) (Dubey and Bwangamoi, 1994). Other than descriptive morphology of sarcocysts, little is known about *S. felis*; no molecular or biological studies have been reported to date, and the definitive host of this organism is unknown.

In 1990, there were an estimated 60 million feral cats in the United States (Coleman et al., 1993). An APPMA® 2001-2002 National Pet Owners Survey[1] indicated there were 73 million owned cats in the United States. Beyond the laboratory setting, only one cat has been reported to have *S. neurona* sarcocysts, and this infection was not fully characterized (Turay et al., 2002). Because investigations into the relationship between cats and *S. neurona* are recent and limited in scope, more information is needed about the cat's role as a natural intermediate host for *Sarcocystis* species. Cats are frequently found around horse barns, so there is concern that cats should be controlled if they are involved as hosts in the life cycle of *S. neurona*. If, however, cats are not an important intermediate host for this *Sarcocystis* species, the unnecessary removal of large numbers of cats can be avoided.

This thesis examines *Sarcocystis* spp. infections in domestic cats. The objective of the study is to determine if cats are important, natural hosts for *S. neurona*. This objective will be achieved by examining cat muscle for the presence of sarcocysts, characterizing these sarcocysts, and evaluating cat sera for the presence of *S. neurona* antibodies. The study hypothesis maintains that cats are not important, natural hosts for *S. neurona*.

This is the first survey to evaluate relatively large numbers of domestic cats for the presence of sarcocysts, and the first to test for *S. neurona* antibodies by criteria used for commercial-scale testing of horse sera and cerebrospinal fluid (CSF) for EPM. Sarcocysts found in naturally-infected cats from this study were characterized by morphologic, molecular, and biological methods. These data were compared to data from other studies evaluating cats as hosts for *Sarcocystis* species.

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CHAPTER 2
LITERATURE REVIEW

Sarcocystis

The name *Sarcocystis* describes a specific group of obligate intracellular protozoan parasites. The name is Greek, derived from *sarkos,* meaning muscle or flesh, and *kystis*, meaning bladder, and describes the terminal asexual life stage of the parasite found encysted in the tissue of its host (Dubey et al., 1989, Dubey and Odening, 2001). The genus *Sarcocystis* was named by Lancaster in 1882, however, the parasite was described 40 years earlier by Miescher (Dubey et al., 1989, Dubey and Odening, 2001). *Sarcocystis* spp. are found in a large variety of hosts (wild animals, domestic animals, and man) and are found worldwide. One unique characteristic of *Sarcocystis* is that it has a diheteroxenous life cycle; two different types of host, a definitive host and an intermediate host, succeed one another in the life cycle (Odening, 1998). These different hosts accommodate different life stages of the parasite; sexual reproduction occurs in the definitive host, and asexual reproduction in the intermediate host (Dubey et al., 1989, Dubey and Odening, 2001). Definitive hosts are carnivorous, typically infected by preying or scavenging on infected intermediate hosts. *Sarcocystis* may use one or more similar types of intermediate host and/or definitive host species, although some *Sarcocystis* spp. use several unrelated intermediate hosts (or definitive host animals) in their life cycle (Dubey et al., 1989, Odening, 1998). Currently there are 189 named species of *Sarcocystis*; undoubtedly more species will be discovered and named in the

future. Both definitive and intermediate hosts are known for only 89 species (Odening, 1998).

Taxonomy

Sarcocystis parasites belong to the phylum Apicomplexa, class Sporozoasida, order Eimeriorina, family Sarcocystidae, and genus *Sarcocystis* (Dubey and Odening, 2001). Variations of this categorization, beginning at the family level and above, are recognized as some parasitologists use subcategories, while others do not (Urquhart et al., 1996). As Apicomplexans, *Sarcocystis* spp. possess the characteristic apical complex, located at the anterior end of certain life stages, that is thought to aid in cell penetration (Dubey and Odening, 2001). Genera related to *Sarcocystis* include *Eimeria*, *Isospora, Cryptosporidium, Toxoplasma, Neospora, Hammondia, Besnoitia,* and *Frenkelia*. These organisms are biologically distinct from *Sarcocystis*; location and structure of specific life stages differ. Most closely related to *Sarcocystis* are: *Toxoplasma, Neospora, Hammondia, Besnoitia,* and *Frenkelia*, all cyst-forming coccidians capable of using two vertebrate hosts to complete their life cycle (Urquhart et al., 1996).

Host species names are commonly used to name and classify *Sarcocystis* species, for example *S. capracanis*, and *S. suihominis* (Dubey et al., 1989, Odening, 1998). However, it is now known that one species of *Sarcocystis* may be present in several species of hosts and one species of host may harbor several *Sarcocystis* species. Now, the ultrastucture of the sarcocyst wall is used for the taxonomic classification of *Sarcocystis* species in preference to host species names (Dubey et al., 1989, Dubey and Odening, 2001). The structure of the sarcocyst wall may indicate phylogenetic relationships among hosts; for instance *Sarcocystis* species found in sheep and goats all possess similar cyst wall structure (Dubey et al., 1989). Currently there are 37 sarcocyst wall types used to

distinguish species. Ultrastructural cyst wall type is known for 119 species (63%) (Odening, 1998).

Life Cycle

Sarcocystis has a diheteroxenous, two-host life cycle in which the definitive host is infected by eating the sarcocyst-infected muscle or neural tissue of an intermediate host. The definitive host is carnivorous and the intermediate host is typically a herbivorous prey animal. However, some definitive host species prey upon other carnivores or scavenge from dead animals, allowing for both herbivorous and carnivorous intermediate hosts, including birds and reptiles (Dubey et al., 1989). Some argue that the life cycle of several *Sarcocystis* spp. can also be called dihomoxenous; in rare cases, definitive and intermediate hosts belong to the same species. For instance, mice can function as both definitive and intermediate hosts for *S. muris* or *S. rodentifelis* (Šlapeta et al., 2001). Key however, is that different stages occur in separate animals, even if they belong to the same species (Odening, 1998). *Sarcocystis gallotiae* represents a unique case. The life cycle of this *Sarcocystis* spp. has been called monoxenous in that different life stages occur not only in the same species, but possibly the same animal; the host, a Canary Island lizard named *Gallotia galloti,* eats its own tail, ingesting sarcocysts in the process (Matuschka and Bannert, 1987).

When compared to related parasites, several features of the life cycle of *Sarcocytis* are unique. Asexual reproduction, schizogony and sarcocyst formation, takes place only in the intermediate host. Bradyzoites represent a state of “arrested development”; no further development occurs until they are ingested by a definitive host, where they then develop into gametocytes rather than schizonts. Sexual reproduction occurs only in the definitive host and sporogony is accomplished fully within the definitive host; no further

development of sporocysts occurs once they are passed in the definitive host's feces (Dubey, 1989).

The following details from the life cycle of *Sarcocystis* spp. have been summarized from the text "Sarcocystosis of Animals and Man" by Dubey et al., 1989.

Digestion of sarcocysts releases bradyzoites that invade the small intestinal epithelium of the definitive host. There, bradyzoites undergo gametogony either immediately, or after a period of several days (species dependent), differentiating into both micro (male) and macro (female) gamonts; many more macrogamonts are formed than are microgamonts. As the microgamont matures, its nucleus divides multiple times; the resultant nuclei move to the margin of the microgamont, and differentiate into microgametes that are released and disperse, aided by two flagella, to fertilize macrogametes. Postfertilization, a thin-walled oocyst is formed. Oocysts undergo asynchronous sporulation, resulting in two sporocysts, each containing four sporozoites. Oocysts or sporocysts are passed in the feces, but are also infective even before release from the small intestinal epithelium. The sexual reproduction process can be completed in 24 hours and is asynchronous; both gamonts and oocysts are found simultaneously.

Intermediate hosts ingest sporocysts from fecal-contaminated food or water. Aided by bile salts, excystation of sporocysts occurs in the intestinal tract, releasing sporozoites. Sporozoites enter the small intestinal epithelium and migrate to the endothelial cells of the mesenteric lymph node arteries. One or more rounds of asexual multiplication, called schizogony (or merogony), occur here. Further rounds occur throughout the body in blood monocytes and vascular epithelium. The location and number of rounds of schizogony is species-dependent. Schizonts multiply by endopolygeny; numerous

merozoites develop simultaneously within the schizont and bud at its surface. Merozoites from second (or succeeding) generation schizonts invade striated muscle, or in some cases nerve cells, and form sarcocysts. The formation and maturation of a sarcocyst is initiated when the merezoite becomes surrounded by parasitophorous vacuole and develops a parasitophorous vacuolar membrane. The merozoite then divides by endodyogeny, producing two metrocytes. Metrocytes rapidly divide, again by endodyogeny, producing many bradyzoites within the sarcocyst. The parasitophorous vacuolar membrane transforms into the primary cyst wall. Sarcocysts mature over one to several months to become infective for the definitive host, and are the terminal asexual stage, primarily found in skeletal muscle, heart, tongue, esophagus, and diaphragm of the intermediate host. Factors affecting the number and distribution of sarcocysts include the number of sporocysts ingested, species of *Sarcocystis*, species of host, and the immunological status of the host.

Morphology

Sarcocysts vary in size and shape depending on species and age. Some species are always microscopic, for example *S. cruzi*, whereas others become macroscopic, i.e. *S. gigantea*, and *S. muris*. Common shapes are filamentous, elongated, or globular. Shape and size of sarcocysts is also dependent upon the type of host cell they are contained in; long, slender cells contain long, slender sarcocysts. Macroscopic sarcocysts are nearly always in skeletal muscle or esophageal muscle (Dubey et al., 1989). Because they are derived from a merozoite having distinct anterior and posterior shapes, sarcocysts often retain anterior and posterior features as well (Odening, 1998). The sarcocyst consists of a cyst wall that surrounds metrocyte or bradyzoite stages within. The structure and thickness of the cyst wall varies among *Sarcocystis* species; this feature is now the

primary means of differentiating species. A connective tissue capsule, formed by the host, surrounds the cyst wall of fourteen species (Odening, 1998), as in *S. gigantea*.

Ultrastructural sarcocyst wall morphology is classified by type based upon a system imposed by Dubey et al. (1989); 24 specific types have been described in detail. Recently, an additional 13 types have been described (Dubey and Odening, 2001), bringing the total to 37. Size, shape and spacing of cyst wall villi, and the presence or absence of microtubules within the villi, constitute this system of classification. The sarcocyst wall, ground substance underlying it, and the septae are remnants of the merozoite. The metrocytes and bradyzoites within the sarcocyst are formed from endodyogenic divisions of the merozoite nucleus. Other than the sarcocyst wall, ultrastructural morphology is fairly consistent among species; a few species lack septae, and the density of bradyzoites within the sarcocysts may vary by species (Dubey and Odening, 2001). Mature sarcocysts may contain both metrocytes and bradyzoites, although bradyzoites predominate (Dubey et al., 1989). Bradyzoites are surrounded by three membranes, forming the pellicle, and have distinct anterior and posterior ends. At the anterior end are the apical complex and numerous micronemes and several rhoptries. Micronemes are thought to differentiate into rhoptries; both appear by transmission electron microscopy (TEM) as dark structures, and are believed to secrete products that aid in cell penetration (Dubey et al., 1989). Numbers of rhoptries in each bradyzoite may differ among *Sarcocystis* species. Although bradyzoites and metrocytes contain the same typical cell organelles such as a nucleus, endoplasmic reticulum, mitochondria, inclusion bodies, etc., metrocytes lack the distinctive apical complex, micronemes, and rhoptries found in bradyzoites (Dubey et al., 1989).

When compared to the sarcocyst, other life stages of *Sarcocystis* are of little value in distinguishing among species, although sporocyst size may be useful (Cheadle et al., 2001c); a typical sporocyst measures 12 μm X 10 μm. Dubey and colleagues, again in their "Sarcocystosis of Animal and Man" text (1989), describe the morphology of the various *Sarcocystis* life stages. Sporocysts are surrounded by two membranes, the inner composed of four fused plates, and contain four sporozoites and a residual body. Sporozoite morphology is very similar to that for bradyzoites. Once inside the intestinal epithelial cells of the intermediate host, sporozoites differentiate into schizonts; unlike sarcocysts and merozoites, a parasitophorous membrane does not surround sporozoites and schizonts within host cells. Schizonts change morphologically as they mature and divide by endopolyogeny; the nucleus becomes multi-lobed, each lobe giving rise to two merozoites. Merozoites vary in size and shape but are typically reported to be approximately 8 μm X 2.5 μm. Merozoites also possess anterior and posterior ends and an apical complex, but do not contain rhoptries.

Gametogony in the definitive host produces rounded macrogamonts, 10-20 μm in diameter, and elongated microgamonts, approximately 7 X 5 μm. Microgamonts become multi-nucleated, and eventually contain 3 to 11 slender, bi-flagellated, microgametes typically measuring 4 X 0.5 μm. After fertilization, the zygote differentiates into an oocyst, approximately 24 X 20 μm and containing two sporocysts within.

Pathogenesis

Typically there is little or no illness in the definitive host other than gastrointestinal disease (Dubey et al., 1989, Urquhart et al., 1996, Dubey and Odening, 2001). However, the pathogenicity of *Sarcocystis* spp. in intermediate hosts varies by species, from

mildly-pathogenic (*S. fayeri* in horses, *S. gigantea* in sheep) to very pathogenic (*S. cruzi* in cattle, *S. capricanis* in goats), and host; disease in small intermediate hosts, i.e., *S. falcatula* in some bird species, and *S. idahoensis* in deer mice, is more severe than that seen in large animal hosts (Dubey et al., 1989). In experimental infections, severity of host disease was dependent upon number of sporocysts given (Dubey et al., 1989). As *Sarcocystis* spp. are obligate intracellular parasites, disease is caused by the destruction of host cells; hemorrhage and inflammatory lesions are commonly seen (Dubey et al., 1989, Dubey and Odening, 2001). Infection of the central nervous system occurs only rarely with most *Sarcocystis* spp. (Dubey et al., 1989) except for *S. neurona*, which causes neurological disease in several animal species. In horses, equine protozoal myeloencephalitis (EPM), discussed in the next section, is the result of such an infection. In food animals, the presence of sarcocysts, caused for example by *S. gigantea*, can result in condemnation of meat, resulting in economic losses for farmers (Dubey et al., 1989). *Sarcocystis* infection (ex: *S. cruzi*, *S. ovicanis*) has also been implicated in abortions in farm animal hosts (Dubey et al., 1989, Urquhart et al., 1996).

Equine Protozoal Myeloencephalitis (EPM)

Considerable research effort has been devoted to one species of *Sarcocystis*, *Sarcocystis neurona*, due to the fact that *S. neurona* infection in horses is the principle cause of equine protozoal myeloencephalitis (EPM) (Dubey et al., 1991). EPM is seen only in the Americas, a fact that puzzled researchers until it was found that the distribution of EPM follows that of the definitive host, the opossum. Both the Virginia opossum (*Didelphis viginiana*) and the South American, white-eared opossum (*Didelphis albiventris*) are definitive hosts of *S. neurona* (Fenger et al., 1995, Dubey et al., 2001e). Although now likely superceded by West Nile Encephalitis, in 2001, EPM was the most

commonly-diagnosed neurological disease of horses in the United States (Dubey et al., 2001d). It is estimated that EPM has caused greater than $100 million in losses to the nation's equine industry (Dubey et al., 2001d). Regarding clinical EPM, costs associated with diagnosis and treatment alone range from $55.4 to $110.8 million per year (Dubey et al., 2001d).

Signalment

Clinical signs of EPM can be variable due to the location and severity of parasite damage to the CNS (MacKay et al., 2000). Common signs include head tilt, facial paralysis, difficulty swallowing, depression (all due to brain or brain stem damage) and ataxia and gait abnormalities (due to spinal cord damage). Another clinical sign, focal muscle atrophy, is often characteristic of EPM. Clinical signs may worsen suddenly, or improve, although relapse is common; EPM is typically a progressive disease (MacKay et al., 2000). Related parasites from the genus *Neospora* are implicated in several cases of EPM. *Neospora hughesi* is proposed as the causative agent (Marsh et al., 1998).

History

In the review of EPM by MacKay and co-authors, (2000), the recognition and emergence of EPM is described. Neurological syndromes designated "segmental myelitis" and "focal encephalitis-myelitis" were identified in the 1960's (Rooney et al., 1970). In 1974, protozoal organisms were found to be associated with these lesions, and were thought to be *Toxoplasma gondii* (Cusick et al., 1974). Another report attributed the infections to a *Sarcocystis* species (Dubey, 1976), later confirmed by electron microscopy in 1980 (Simpson and Mayhew, 1980). The name "equine protozoal myeloencephalitis" was proposed in 1976 by Mayhew et al. and widely adapted. In 1991, the causative agent was named "*Sarcocystis neurona*"(Dubey et al., 1991).

Epidemiology

EPM affects primarily young horses; an analysis of histologically-confirmed cases of EPM found that most, 62%, were 4 years old or less (Fenger, 1997). Diagnosis by neurological examination alone found young horses (1-5 years old), and older horses (>13 years old) were at an increased risk of developing EPM (Saville et al., 2000a). In addition to age, there are other risk factors for EPM. Immune suppression, linked to overall health, advancing age, and stress is associated with an increased incidence of EPM (Saville et al., 2000b). Because of stress, show and racehorses are affected more than horses kept for breeding or pleasure (Saville et al., 2000a). By neurological examination, more cases of EPM were diagnosed in summer and fall than were in other seasons (Saville et al., 2000a), possibly reflective of increased exposure to host animals, increased survival of *S. neurona* in the environment (Saville et al., 1997), or stress induced by timing of competitive events (MacKay et al., 2000). Horses were also more at risk for developing EPM when opossums were present in the environment or there was close proximity of opossum habitat, and if rats and mice, possible hosts, were present in the environment (NAHMS, 1998).

Exposure to *S. neurona*, as measured by the presence of *S. neurona* serum antibodies, indicates that approximately 50% of horses in the United States have been exposed (MacKay et al., 2000). However, the prevalence of EPM, based upon postmortem evaluation, is estimated to be less than 1% (Granstrom, 1997, NAHMS, 1998).

Clinical Diagnosis

Diagnosis of EPM can be made by antemortem or postmortem methods. At necropsy, gross lesions of the CNS are sometimes visible in horses afflicted with EPM;

dark hemorrhagic discolorations of spinal cord sections are frequently depicted. Parasites are not always found in CNS lesions; only 10 to 36% of hematoxylin and eosin preparations and 20 to 51% of immunohistological preparations revealed parasites (MacKay et al., 2000). However, characteristic and consistent lesions are often seen in preparations when parasites are absent. These lesions include perivascular cuffing by mononuclear cells, infiltrates of lymphocytes, neutrophils, eosinophils, multinucleate giant cells, and astrocyte proliferation (MacKay et al., 2000, Dubey et al., 2001d).

Antemortem diagnosis of EPM is best made by a thorough examination of the horse and suitable clinical laboratory tests. Differentiating EPM from other neurological diseases can sometimes be difficult. Other diseases to consider include: cervical vertebral malformation, equine herpesvirus-1 myeloencephalopathy, equine motor neuron disease, tumors, abscess, migrating metazoan parasites, rabies, West Nile viral encephalitis, equine degenerative myeloencephalopathy, and vascular and bone malformations (MacKay et al., 2000).

Immunological Diagnosis

An immunoblot test to detect *S. neurona* IgG in serum or CSF is used as an aid to the diagnosis of EPM. A positive immunblot test of CSF from a neurologic horse suggests CNS infection, intrathecal production of *S. neurona* antibody, and EPM. Collection of CSF must be done by a qualified veterinarian; the procedure poses some risk to both horse and veterinarian, and sample quality is very important. The procedure can also be expensive to perform (Dubey et al., 2001d).

The immunoblot test has evolved to some extent since its development in the early 1990's. Initially, eight proteins, of varying molecular weight, were identified as *S. neurona* specific (Granstrom et al., 1993). Cross-reactive *S. fayeri*, *S. cruzi*, and

S. muris proteins were identified by this test and excluded in the interpretation of positive results. Versions of this test are currently commercially used for EPM testing at three laboratories: Neogen Corporation (Lexington, KY), Equine Biodiagnostics Incorporated (EBI, Lexington, KY), and the Michigan State Animal Health Diagnostic Laboratory, (East Lansing, MI). Each of these laboratories offers slightly different test formats and therefore, interpretation of results. The Michigan State test, for example, now preadsorbs blots with *S. cruzi*-positive bovine serum to increase test sensitivity and specificity. Due to differences in test format, reagents, parasite preparations, etc., positive results are interpreted differently among labs. Serum or CSF reacting against a 17-kDa protein of *S. neurona* is considered positive at the Neogen EPM testing lab. At EBI, reactivity against 14.5, 13, and 7-kDa proteins constitutes a positive result. At Michigan State, reactivity against both 30-kDa and 16-kDa proteins must be present for a positive result.

Granstrom (1997) describes the use of a non-commercial immunoblot in a population of 295 horses euthanized for neurological disease. Sensitivity of the test, the ability of the test to detect antibody in CSF from EPM-positive horses, was 89% (11% false negative). Specificity, the ability of the test to give negative results for EPM-negative horses, was also 89% (11% false positive). False positive results can result from defects in the blood-CNS barrier, or more commonly, from blood contamination of the sample. The positive predictive value of this test, those horses testing positive that are truly positive in that population was 85%. The negative predictive value, the percentage of horses testing negative that are truly free of EPM was 92%. However, because the prevalence of EPM is estimated to be only approximately 1%, when this test was used in a normal population of horses (instead of the neurologic horses tested) the positive

predictive value of the test drops to 8%; a test's predictive values are dependent upon the prevalence of the disease in the population tested. For this reason, the authors suggest that the immunoblot test not be used for screening CSF from normal horses. From the same study, serum testing yielded a test sensitivity of 89% (11% false negative) and a specificity of 71% (29% of neurologic horses did not have EPM, but did have antibody) confirming the accepted premise, even within this group of neurological horses, that serum samples testing positive show merely that the horse has been exposed to *S. neurona*. Approximately 50% of horses in the U.S. have serum antibodies to *S. neurona*, while only 1% have EPM (MacKay et al., 2000).

Immunoblots of CSF and serum from both neurologic and clinically-normal horses were evaluated by Daft et al. (2002). Immunological results were compared to immunohistochemistry postmortem. The sensitivity of the immunoblot used in the study was high, 80%-88%, for both serum and CSF from either neurologic or clinically-normal horses. However, the specificity was lower: for CSF, 44% for neurologic horses, and 60% for normal horses, and for serum, 38% for neurologic horses, and 56% for normal horses. False positive results were decreased when weakly reacting or indeterminate samples were categorized as negative. Daft and co-authors state that the lower specificity of the test in neurologic horses may reflect damage to the blood-CNS barrier in horses with neurologic disease other than EPM, or, as indicated previously, these horses could have been exposed to *S. neurona* and have a neurologic disease other than EPM. Daft and coauthors report 40% of horses negative for EPM, had *S. neurona* antibodies in the CSF, concluding that weakly-positive CSF may be normal for some seropositive horses. The

value of the test therefore is in ruling out EPM; a negative result is more useful than a positive one.

Despite its limitations, the wide acceptance of the immunoblot for detection of *S. neurona* antibody in horses has meant that other immunological methods, such as indirect fluorescent antibody (IFA) testing or enzyme-linked immunosorbent assay (ELISA) testing, are so far, confined to research applications. The use the polymerase chain reaction (PCR) for detection of *S. neurona* in CSF is not yet optimized; the sensitivity of this method is not as great as hoped (MacKay et al., 2000). This may be due to the scarcity of parasite in the CSF, or the lack of intact DNA. The use of PCR testing may be helpful when *S. neurona* antibody is weak or undetectable in CSF from a horse with neurologic signs consistent with EPM (Dubey et al., 2001d).

Treatment

Horses that receive treatment for EPM were 10 times more likely to improve, and those that improve were 50 times more likely to survive (Saville, 2000a). If started early after the onset of clinical signs, treatment may be effective in up to 75% of cases (Dubey et al., 2001d). Traditional EPM treatment involves the use of folate inhibitors such as sulfadiazine/pyrimethamine combinations. Horses are often treated long-term with this combination, for months at a time, or even indefinitely. Side effects such as anemia and leucopenia have been noted (MacKay et al., 2000). Anti-coccidial triazine compounds such as diclazuril, toltrazuril, and its sulfone metabolite ponazuril, are relatively new EPM therapies (MacKay et al., 2000). Currently, ponazuril (Marquis®, Bayer Animal Health, Shawnee Mission, KS) is the only product approved specifically for such use. Triazines must also be adiminstered for weeks at a time (28 days at 5mg/kg for ponazuril), and are very costly, up to $1000.00 for a course of treatment. Another

medication, nitazoxanide, is a coccidiocidal therapeutic agent currently under review by the FDA for the treatment of EPM. There have been reports of toxicity associated with nitazoxanide; however, these effects were lessened when dosages were reduced (MacKay et al., 2000). As with other EPM treatments, nitazoxanide therapy may be lengthy and costly.

Prevention

Prevention of EPM has focused on limiting the access of opossums to feed, hay, water, and bedding supplies. Some horse owners have chosen to feed only pelleted feeds that have gone through a heat process killing sporocysts which may be present on feedstuffs or feed ingredients (MacKay et al., 2000). Opossums are sometimes trapped on horse farms and moved to other locations or killed (personal communication, T. J. Cutler).

Recently, an EPM vaccine, "designed to aid in the prevention of neurologic disease due to subsequent infections of S. neurona" (Ft. Dodge Animal Health, Ft. Dodge, IA) has been introduced. The vaccine is under conditional license from the United States Department of Agriculture (USDA); results from efficacy studies, currently in progress, must be satisfactory before full licensure is granted. The vaccine is manufactured from S. neurona merozoites harvested from cell culture; standardized numbers of merozoites are chemically-inactivated and mixed with adjuvant. One concern regarding the use of this vaccine is that serum and CSF samples may test immunoblot positive, particularly when tested shortly after vaccination, reducing the value of a negative CSF immunoblot (Fort Dodge Animal Health Bulletin http://www.epmvaccine.com/technical/07Effects.htm).

Sarcocystis neurona

Named in 1991 for the *Sarcocystis* spp. isolated from spinal cord lesions of horses afflicted with EPM (Dubey et al., 1991), *S. neurona* has now been characterized to some extent by molecular, biological, and morphological methods. These research efforts aim to differentiate *S. neurona* from other *Sarcocystis* species, identify other possible animal hosts for this parasite, and improve our understanding of EPM. *Sarcocystis neurona* was known to exist before its definitive and intermediate hosts were identified. Morphologic analysis of merozoites and schizonts isolated from CNS lesions from several horses with EPM showed that the same parasite was present in each of these lesions, and that it was a distinct species of *Sarcocystis* (Dubey et al., 1991, Dubey et al., 2001d). *Sarcocystis neurona* was then successfully cultured in vitro (Davis et al., 1991a, 1991b), allowing for more comprehensive characterization.

Life Cycle

Fenger and colleagues (1995) proposed the Virginia opossum (*Didelphis virginiana*) as a definitive host because of its status as an omnivore/carnivore endemic in areas where EPM was found in horses; the study also then provided molecular evidence that sporocysts shed by opossums were *S. neurona* sporocysts. The definitive host status of the opossum was confirmed by Fenger et al., in 1997, when opossum sporocysts administered to horses induced *S. neurona* antibody and neurologic disease in these horses. Another opossum species, *Didelphis albiventris*, the South American white-eared opossum was also found to be a definitive host for *S. neurona* (Dubey et al., 2001e). Other species of *Sarcocystis* sporocysts are passed by the opossum as well and can be can be differentiated by molecular (Tanhauser et al., 1999,Dubey et al., 2001f, Rosenthal et al., 2001), and possibly morphological, methods (Cheadle et al., 2001c).

The life cycle of *S. neurona* is completed when the opossum eats sarcocyst-infected muscle from an intermediate host, most probably from scavenging road-killed host animals. The intermediate host(s), and thus the complete life cycle were unknown for a decade or more since the discovery and isolation of *S. neurona*. In 2000, the life cycle was completed in the laboratory with domestic cats as experimental intermediate hosts (Dubey et al., 2000). Shortly thereafter, the armadillo (*Dasypus novemcinctus)*, striped skunk (*Mephitis mephitis*), and raccoon (*Procyon lotor*) were identified as intermediate hosts as well (Cheadle et al., 2001a, Tanhauser et al., 2001, Cheadle et al., 2001b, Dubey et al., 2001a). The sea otter (*Enhydra lutris*) was also found to be naturally-infected (Lindsay et al., 2000) and capable of infecting opossums. Sea otters likely become infected by storm water run off contamination of shellfish stocks (Dr. Ellis Greiner, personal communication); their status as natural intermediate hosts for *S. neurona* is unlikely (Dubey et al. 2001b). Once intermediate hosts for *S. neurona* were identified, the sarcocyst stage was then described (Cheadle et al., 2001a, Cheadle et al., 2001b, Dubey et al., 2001a, Dubey et al., 2001b, Dubey et al. 2001c). *Sarcocystis neurona* therefore utilizes a single type of definitive host (although others may be identified in the future) that is host to other *Sarcocystis* species, and multiple intermediate hosts, each known to be hosts for other *Sarcocystis* spp. as well (Odening, 1998).

Pathogenesis

Sarcocystis neurona was named for its ability to infect neural tissue in a dead-end host; no muscle sarcocysts have been found in horses (MacKay et al., 2000). Asexual life stages (merozoites, schizonts, and occasionally sarcocysts) of *S. neurona*, and EPM-like signs, are found in neural tissues of other animals: cats (Dubey et al., 1994, Dubey and Hamir, 2000, Forest et al., 2000), skunks (Dubey and Hamir, 2000), raccoons (Hamir and

Dubey, 2001, Dubey and Hamir, 2000), mink (*Mustela vison*) (Dubey and Hedstrom, 1993, Dubey and Hamir, 2000), zebra, (*Equus burchelli bohmi*) (Marsh et al., 2000), Pacific harbor seal (*Phoca vitulina richardsi*) (Miller et al., 2001a), sea otter (Miller et al., 2001b, Lindsay et al., 2000, Lindsay et al., 2001), Straw-necked ibis (*Carphibis spinicollis*) (Dubey et al., 2001g), and gannet (*Morus bassanus*) (Spalding et al., 2002). Both neural and inflammatory cells in the CNS can become parasitized (Dubey et al. 2001d). Other *Sarcocystis* spp. are found only rarely in the CNS (Dubey et al., 1989). Later reports have identified several of the aberrantly-infected animal species listed above to be intermediate hosts for *S. neurona*; tissue from these animals was capable of infecting opossums when fed in an experimental feeding trial (Dubey et al., 2000, Dubey et al., 2001a, Dubey et al., 2001b, Dubey et al., 2002, Cheadle et al., 2001b).

Diagnosis

Morphological. Morphological diagnosis of *S. neurona* infection has only really been made feasible since the discovery of its intermediate hosts. Ultrastructural characterization by TEM has shown the sarcocyst wall of *S. neurona* as having tapered villar projections and microtubules within villi (Dubey et al., 2000, Dubey et al. 2001b, Dubey et al., 2001c, Cheadle et al., 2001b). Cheadle and colleagues (2001b) note that this morphology correlates to a type 11 per the classification system instituted by Dubey et al. in 1989. However, the same report notes that TEM of the *S. neurona* sarcocyst found in skunks resembles that seen for other *Sarcocystis* species: *S. dasypi*, *S. kirkpatricki* (a *Sarcocystis* spp. seen in a raccoon), S. *falcatula*, and *S. fayeri*. Ultrastructural morphology alone may not be sufficient to differentiate species. Other details seen by TEM include the presence of relatively few rhoptries, and bradyzoites 5 μm long by 1 μm

wide (Dubey et al., 2001c). In the same report, *S. neurona* sarcocysts have measured 700 μm in length by 50 μm in width, and in another report, 140 μm X 20 μm (Butcher et al. 2002). Although not of significance for species diagnosis, Speer and Dubey (2001) have described the ultrastructure of *S. neurona* schizonts and merozoites.

Molecular. Molecular analysis was one of the early tools used to identify and characterize *S. neurona*. Random-amplifed polymorphic DNA (RAPD) markers were developed to differentiate *S. neurona* from other Apicomplexan parasites and some *Sarcocystis* species (Granstrom et al., 1994). Sequencing and PCR of the small subunit ribosomal RNA (ssurRNA) gene prepared from *S. neurona* merozoites was used to identify the Virginia opossum as a definitive host (Fenger et al., 1995). Small subunit ribosomal DNA sequence was then used to conclude *S. neurona* and a similar species, *S. falcatula,* were synonymous, and therefore birds, particularly the brown-headed cowbird (*Molothrus ater*), were likely intermediate hosts (Dame et al., 1995). This conclusion proved erroneous when additional RAPD-based markers were shown to differentiate *S. neurona* from *S. falcatula* and other species found in the opossum (Tanhauser et al., 1999). Additional methods such as infectivity of animal hosts (Cutler et al., 1999) or cell cultures (Lindsay et al., 1999) further differentiated these two *Sarcocystis* species. Comparison of sequences from the internal transcribed spacer 1 (ITS-1) region of ribosomal genes, more rapidly evolving than small subunit genes, were also useful for differentiating *S. neurona* from *S. falcatula* (Tanhauser et al., 1999 and Marsh et al., 1999). The RAPD-derived markers and restriction endonuclease protocols developed by Tanhauser and co-workers (1999) are now widely used to identify *S. neurona* in both new intermediate hosts and in aberrantly-infected animals. *Sarcocystis*

neurona sequence data from loci amplified by these markers, as well as other regions of ribosomal RNA gene sequences have been archived in GenBank™ and are proving useful for phylogenetic analyses of *Sarcocystis* species.

Immunological. Immuno-based methods are also used to identify *S. neurona* exposure or infection and characterize antibody response in the infected host(s). Besides their use in horses, discussed in the previous section for diagnosis of EPM, serologic surveys have been used to aid in the identification of natural intermediate hosts for *S. neurona* (Tanhauser et al., 2001, Stanek et al., 2001, Rossano et al., 2002, Turray et al., 2002, Mitchell et al., 2002). Several methods of serologic examination have been used: direct agglutination testing (Dubey et al., 2001a, Lindsay and Dubey 2001, Dubey et al., 2000, Dubey et al, 2002, Dubey et al., 2001b), indirect fluorescent antibody testing (Lindsay et al., 2000, Miller et al., 2001a, Butcher et al., 2002, Ellison et al., 2002, Rossano et al., 2002), and immunoblotting (Cheadle et al., 2001a, Cheadle et al., 2001b, Tanhauser et al., 2001, Turay et al., 2002, Butcher et al., 2002, Rossano et al. 2002, Long et al., 2002). Immunohistochemistry is also widely used, demonstrating the presence of *S. neurona* schizonts in the CNS of aberrant and experimental hosts (Dubey et al., 2000, Lindsay et al., 2000, Dubey et al., 2001a, Dubey et al., 2001b, Cheadle et al., 2001b, Lindsay and Dubey 2001, Turay et al., 2002, Butcher et al., 2002, Fritz and Dubey, 2002). Sections of brain tissue are usually examined by this method. However, identification by immunohistochemistry relies on the use of polyclonal *S. neurona* antiserum, so infection in these animals is presumptive unless followed by additional diagnostics (Dubey et al., 2001g, Dubey et al, 2001d). In addition to cross-reactivity across *Sarcocystis* species, immunohistochemistry has also demonstrated cross-reactivity

across different strains (i.e., horse spinal cord-derived or intermediate host-derived) and life stage of the same species (Turay et al., 2002, Butcher et al., 2002). Such variable immunoreactivity has meant that other techniques, such as biological assay, molecular characterization, or morphologic identification, are typically used in conjunction with immunological methods to confirm *S. neurona* infection.

Biological. *Sarcocystis neurona* has been successfully cultured in a variety of cell lines; bovine monocytes (Dubey et al., 1989), equine dermal cells (Murphy and Mansfield, 1999), and bovine turbinate cells (Speer and Dubey, 2001) are commonly used for this purpose. Cultures have been established from merozoites (Dubey et al., 2001d) and sporocysts (Murphy & Mansfield, 1999). *Sarcocystis* species can be differentiated biologically from related Apicomplexans by their behavior *in vitro* (Dubey and Odening, 2001). *Sarcocystis neurona* does exhibit certain characteristics in culture (Lindsay et al., 1999), but these characteristics have not been reported as a means to differentiate it from other *Sarcocystis* species. The availability of culture-grown isolates, however, has facilitated efforts to more fully characterize *S. neurona*.

In vivo methods, i.e. host specificity and location of infection (aberrant infections with *S. neurona* are confined to the CNS) (Dubey et al., 1989, Dubey et al., 2001d) have been used to diagnose *S. neurona* infection. Now, other *in vivo* methods are routinely for diagnosis and characterization *S. neurona* infection. An animal model of infection for studies of the pathogenesis of *S. neurona* was developed in immune-deficient mice (Marsh 1997, Dubey and Lindsay, 1998). Both nude and gamma interferon knockout mice can be inoculated either orally, subcutaneously, or intraperitoneally with sporocysts or merozoites of *S. neurona*; these mice develop encephalitic CNS signs within seven to

thirty days postinoculation. Immune-competent mice such as Balb/c, or C57/Bl are not susceptible to *S. neurona* infection. Sporocysts or merozoites from other *Sarcocystis* species are not infective to nude and gamma interferon knockout mice. This infection model can therefore be used to test for the presence of viable *S. neurona* parasite in preparations of host feces, infected tissues, or cultures established from suspected natural or aberrant hosts (Dubey et al., 2000, Lindsay et al, 2000, Cheadle et al., 2001b. Dubey et al., 2001a, Dubey et al., 2001b, Turay et al., 2002, Butcher et al., 2002, Long et al., 2002). Lack of infection in these mice suggests the sample did not contain *S. neurona*, but perhaps some other *Sarcocystis* species that might be present (Dubey and Lindsay, 1998). Athymic nude mice are thought to be susceptible to infection with protozoal organisms due to a deficiency of T-cells (Marsh, et al., 1997), while gamma interferon knockout mice are unable to activate cytotoxic macrophage activity (Deckert-Schluter, 1998).

Biological assays utilizing controlled feeding trials have been used to demonstrate the presence of *S. neurona* infections in hosts and investigate previously unknown hosts. Infected tissue from a suspected host is fed to opossums shown to be currently non-infected. After a period of time, animals fed the suspect tissue are examined for the presence of *S. neurona* infection. Tissue from the newly-infected host can be fed back to the same original host species to demonstrate a complete life cycle (Dubey et al., 2002). Often times, sporocysts or merozoites produced during the life cycle are inoculated into immune-deficient mice to verify the infective agent is *S. neurona* (Dubey et al. 2000, Dubey et al., 2001a, Dubey et al., 2001b, Cheadle et al., 2001b, Turay et al., 2002, Butcher et al., 2002).

Experimental models of *S. neurona* infection utilizing horses have continued to frustrate EPM researchers. Although horses inoculated with *S. neurona* develop clinical signs and *S. neurona* antibodies, no parasites have been recovered from these inoculated horses (Fenger et al., 1997, Cutler et al., 2001, Saville et al., 2001); Koch's postulates of infection have therefore not been fulfilled in horses. Research on this problem continues.

Sarcocystis in Domestic Cats

Historically, intermediate hosts for *Sarcocystis* species were thought to be herbivores, definitive hosts carnivores, and life cycles perpetuated through predator/prey or scavenger/carrion relationships. Cats are listed as definitive hosts for the following 15 species of *Sarcocystis* (the common name of the intermediate host follows in parenthesis): *S. buffalonis* (water buffalo), *S. cuniculorum* (rabbit), *S. cymruensis* (rat), *S. fusiformis* (water buffalo), *S. gigantea* (sheep), *S. hirsuta* (cattle), *S. leporum* (rabbit), *S. medusiformis* (sheep), *S. moulei* (goat), *S. muris* (mouse), *S. neotomafelis* (woodrat), *S. odoi* (deer), *S. poricifelis* (pig), *S. rodentifelis* (rats, mice), *S. wenzeli* (chicken) (Odening, 1998). Cats become infected by preying or scavenging upon these animals. Compared to other *Sarcocystis* definitive hosts, infected cats shed low numbers of sporocysts (Christie et al., 1976, Dubey et al., 1989); cats may not be very good producers of sporocysts, sarcocysts of feline-transmitted species may be slow maturing, or some animal species are more resistant to *Sarcocystis* infection (Dubey et al., 1989).

Cats are able to function as intermediate hosts for *Sarcocystis* spp. as well. Reports dating back to as early as 1956 (Eisenstein and Innes) identified muscle sarcocysts in domestic cats (Kirkpatrick et al., 1986, Everitt et al., 1987, Hill et al., 1988, Edwards et al., 1988, Fiori and Lowndes, 1988) and wild felids. Among the latter group are bobcats (Anderson et al., 1992, Dubey et al., 1992), lions (Bhatavdekar and Purohit, 1963, Dubey

and Bwangamoi, 1994, Kinsel et al., 1998), cheetahs (Briggs et al., 1993), and Florida panthers and cougars (Greiner et al., 1989).

Prevalence of Sarcocysts

It has been suggested that immunosuppression, resulting from lymphoid neoplasia, corticosteroid therapy, infection with feline immunodeficiency virus (FIV) or feline leukemia virus (FeLV), or inbreeding, might permit aberrant sarcocystosis by an opportunistic organism (Kirkpatrick et al., 1986, Hill et al., 1988, Greiner et al., 1989, Briggs et al., 1993), or extraintestinal infection by a *Sarcocystis* spp. for which the cat serves as a definitive host (Edwards et al. 1988). However, sarcocysts have also been found at in normal, healthy felids (Everitt et al., 1987, Fiori and Lowndes 1988, Greiner et al., 1989, Anderson et al., 1992, Dubey et al., 1992, Kinsel et al., 1998). Although typically found at necropsy, surveys for sarcocysts have been conducted using a limited number of healthy cats. In Indiana, sarcocysts were found in 4 of 12 cats (Everitt et al. 1987), and in Missouri, found in 1 of 9 cats (Turay et al. 2002). In another study, sarcocysts were collected from muscle biopsies of 5 cats, however no data was given regarding prevalence (Fiori and Lowndes 1988). Greater rates of sarcocyst infection have been reported in wild species. Necropsy of four mature lions from Namibia revealed sarcocysts in each (Kinsel et al., 1998). In Florida, 50% of bobcats (Anderson et al., 1992), and 83% of free-ranging Florida panthers and cougars (Greiner et al., 1989) were infected. Sixty-six percent of Arkansas bobcats (Dubey et al., 1992) and 70% of captive born and raised cheetahs (Briggs et al., 1993) had sarcocysts.

Morphology

In both domestic cats and wild felid species, sarcocysts have been described incidental to examination of muscle tissue collected at necropsy from cats afflicted with

neoplasia or other debilitating disease (Bhatavdekar and Purohit 1963, Kirkpatrick et al. 1986, Edwards et al. 1988, Hill et al. 1988, Greiner et al. 1989, Briggs et al. 1993, Dubey and Bwangamoi 1994), and from apparently healthy cats (Everitt et al. 1987, Fiori and Lowndes 1988, Greiner et al. 1989, Anderson et al. 1992, Dubey et al. 1992, Kinsel et al. 1998). In these reports, histologic examination revealed sarcocysts of varying sizes, from 24 μm (Kirkpatrick et al., 1986) to 270 μm (Everitt et al., 1987) in diameter by 24 μm (Everitt et al., 1987) to 2100 μm in length (Dubey et al., 1992). The ultrastructure of these sarcocysts appears nearly identical in all cases, having rounded, irregularly-spaced villi devoid of microtubules, a regularly interrupted electron-dense layer underlying the parasitophorous membrane, and septae separating bradyzoites that measure approximately 10 μm long. It was noted that ultrastructural morphology was inconsistent with descriptions of sarcocysts for which the cat serves as a definitive host (Kirkpatrick et al., 1986).

Sarcocystis felis

In 1992, Dubey and co-workers gave the name *Sarcocystis felis* to the organism which caused sarcocysts in the muscle of bobcats . In that report, it was noted that, based on ultrastructural morphology, these sarcocysts were identical to those found in the domestic cat and in cougars and panthers. Later, *S. felis* was formally identified in cheetahs (Briggs et al., 1993) and lions (Dubey and Bwangamoi, 1994). *Sarcocystis felis* and *S. felis*-like sarcocysts have been reported in felids from North America, Africa (Dubey and Bwangamoi, 1994, Kinsel et al. 1998) and India (Bhatavdekar and Purohit 1963). The definitive host of *S. felis* remains unknown.

Pathogenesis

The pathogenicity of *S. felis* infections in cats is also unknown. The majority of these sarcocysts have been found in skeletal muscle (Kirkpatrick et al., 1986, Everitt et al., 1987, Hill et al., 1988, Fiori and Lowndes, 1988, Greiner et al., 1989, Anderson et al., 1992, Dubey et al., 1992, Briggs et al., 1993, Dubey and Bwangamoi, 1994, Kinsel et al., 1998) and tongue (Greiner et al., 1989, Anderson et al., 1992, Dubey et al., 1992), and occasionally in diaphragm (Kirkpatrick et al., 1986, Greiner et al., 1989, Anderson et al., 1992) and cardiac muscle (Bhatavdekar and Purohit 1963, Kirkpatrick et al., 1986, Everitt et al., 1987, Hill et al., 1988 Greiner et al., 1989, Anderson et al., 1992, Dubey et al., 1992). Only one report of disseminated sarcocystosis is given; this condition was associated with immunosuppression due to lymphosarcoma (Edwards et al. 1988). Rarely described is encephalitis or myelitis in felids caused by infection with *Sarcocystis*; these cases have been attributed to infection of the CNS with *S. neurona* (Dubey et al. 1994, Dubey and Hamir 2000, Forest et al. 2000).

Sarcocystis neurona **in Domestic Cats**

The wide distribution and high prevalence of *S. neurona* antibodies in horses has prompted the search for equally common and widespread intermediate hosts. In 1994, Dubey and co-workers published a report of *Sarcocystis*-associated encephalitis in a domestic cat (Dubey et al., 1994). That infection was later shown to be *S. neurona* (Dubey and Hamir, 2000). As a result, Dubey and colleagues investigated the cat as an intermediate host for *S. neurona* (Dubey et al., 2000). That study provided the first evidence for domestic cats as hosts for *S. neurona*; the life cycle of *S. neurona* was completed through cats and opossums in an experimental bioassay.

Bioassays for *Sarcocystis neurona* in Cats

Sarcocyst formation was induced in four of five laboratory-raised cats dosed with 100,000-250,000 *S. neurona* sporocysts obtained from one naturally-infected opossum. Three of these cats were immunosuppressed by intermittent corticosteroid therapy; one cat not receiving corticosteroids developed sarcocysts. Sarcocysts that formed in cat muscle were infective for four of four juvenile opossums; 250 g of cat muscle was fed to each opossum. The opossums were killed 14 days after eating the infected cat muscle. Sporocysts recovered from the opossums caused *Sarcocystis*-associated encephalitis in 12 gamma interferon knockout mice dosed orally with 250,000 sporocysts each. Mouse sera tested positive for *S. neurona* antibodies by the *S. neurona* agglutination test (SAT). Sarcocysts recovered from the cats were small (700 μm or less) and none were mature; they were composed primarily of metrocytes at 144 days postinfection. Sarcocyst morphology showed slender villar projections containing microtubules. Bradyzoites within the sarcocyst were 5 to 7 μm long. Molecular analysis at the 25/396, and 33/54 loci (Tanhauser et al., 1999) of merozoite rRNA genes recovered from the brain of one cat were consistent with *S. neurona*.

Turay and colleagues (2002) found *S. neurona* antibodies by immunoblot in one of nine feral cats tested from Missouri. Muscle sarcocysts were detected, but not described morphologically, in this single, seropositive cat. Muscle from the cat was fed to a juvenile, laboratory-reared opossum, which then shed low numbers of sporocysts (~5000 total) 17 days postinfection that were infective for two, gamma interferon knockout mice dosed with 300-500 sporocysts each. Merozoites isolated from the mice were used to establish an *in vitro* culture. By immunoblot, antigenic variation among isolates of

S. neurona was demonstrated; serum from the infected cat reacted differently with antigen prepared from each of two horse-derived *S. neurona* isolates. Molecular data from the 25/396 and ITS-1 loci of the cat (opossum, mouse)-derived isolate showed it to be similar to that from *S. neurona*. Because feral cats were evaluated, this study suggested domestic cats might be naturally-infected with *S. neurona*.

In another set of laboratory bioassays, Butcher et al., 2002, characterized infections induced in cats inoculated with horse-derived (UCD 1) or cat (opossum, mouse)-derived (Mucat 2) isolates of merozoites harvested from cell cultures. Intravenous inoculations of 1×10^7 merozoites of either UCD 1 or Mucat 2 were given to two young cats. Two similar cats each received 5×10^7 of the respective isolates by combined intravenous, subcutaneous, and intramuscular route. Three gamma interferon knockout mice were each injected intraperitoneally with 4×10^6 of either the UCD 1 isolate or the Mucat 2 isolate to ensure that the inoculums given to cats were viable. Cats were terminated six to seven weeks postinoculation. Each of five, laboratory-raised opossums received tissue corresponding to an individual cat. Results from this study demonstrated biological diversity between the Mucat2 isolate and the UCD 1 isolate. Two of the cats had developed sarcocysts; each of these cats was inoculated with Mucat 2. One opossum shed a small number (~200) of sporocysts 23 days postfeeding; this opossum received cat muscle from a cat inoculated with Mucat 2. Mice inoculated with Mucat2 merozoites or Mucat2 (opossum) sporocysts developed encephalitis. The UCD 1 isolate did not appear to be biologically active. In addition to the data provided for biological diversity of isolates, this study established that life-cycle stages other than sporocysts of (presumed) *S. neurona* could be infective to cats.

A series of experiments evaluating domestic cats as hosts for *S. neurona* was reported in a single publication by Dubey and colleagues in 2002 (Dubey et al., 2002). Sera collected from cats in the first life-cycle completion study (2000) were evaluated by SAT at 0, 7, 20, 35, 49, and 144-167 days postinfection. For the next experiment, sera collected from three cats dosed subcutaneously with 1×10^6 or more merozoites (isolated from the brains of gamma interferon knockout mice that had received sporocysts from naturally-infected opossums) were evaluated at 0, 3, 5, 8, 9, and 14 weeks postinoculation. These cats were killed and muscle tissue was fed to five opossums, two laboratory-raised dogs, and two laboratory-raised cats. Antibodies to *S. neurona* were found in sporocyst-inoculated cats by 21 days postinfection; antibody titers were 5 to 50 times higher in cats that had received corticosteroid therapy. *Sarcocystis neurona* antibodies developed in cats that received merozoites by the subcutaneous route. One of five opossums fed cat tissue shed sporocysts; for the first time it was possible to produce sporocysts derived from culture-grown merozoites. Neither dogs nor cats fed cat muscle shed sporocysts, suggesting cats and dogs are not definitive hosts for *S. neurona*.

Serological Surveys for *S. neurona* Antibody in Domestic Cats

Two serological surveys for *S. neurona* antibody in domestic cats have been reported. The first utilized the SAT to evaluate the seroprevalence of *S. neurona* antibody in 112 sera from cats in Ohio (Stanek et al., 2001). Seventy-six cats were classified as free roaming. The additional 36 cats came specifically from horse farms. *Sarcocystis neurona* antibodies were found in 26 of 112 (23%) of the cats overall, and in 14 of 36 (39%) of the cats from horse farms. Rossano and colleagues (2002) evaluated the prevalence of *S. neurona* antibody in 196 cats that had been previously tested for

Toxoplasma gondii antibodies. Both IFA and immunoblot were used in the study. By immunoblot, 5% of the cats were seropositive. By IFA, 27% were seropositive. The authors report the IFA test was subject to cross-reactivity when serum dilution was low (1:20 or less); better agreement between IFA and immunoblot results was seen as antibody titers increased.

CHAPTER 3
MATERIALS AND METHODS

All animal procedures used were in accordance with protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Collection of Blood and Muscle Samples

Blood and muscle samples were collected from 50 adult cats euthanized at the local animal shelter[2]. Immediately after euthanasia, cats were transported to the College of Veterinary Medicine for processing. The thoracic cavity was opened, and 10 to 15 mL blood was aspirated from the caudal vena cava. Clotted blood was centrifuged, and serum harvested and stored. Tongue, diaphragm, and right quadriceps muscle were excised and each was divided into three equal parts to be fixed in 10% neutral buffered formalin solution, stored frozen at –80 C, or kept fresh at 4 C. In addition, blood samples were collected by jugular venipuncture from 50 adult feral cats admitted to monthly trap-neuter-return clinics [3] held at the University of Florida, College of Veterinary Medicine.

Examination for Sarcocysts

Three portions of formalin-fixed tissue were taken from each muscle sample, paraffin-embedded, sectioned at 5-μm intervals, mounted on slides, and stained with hematoxylin and eosin (H&E). Stained sections were examined for the presence of sarcocysts using a dissecting microscope at a magnification of 20×. When possible,

[1] Alachua County Animal Services, Gainesville, Fla.

[2] Operation Catnip™

transverse and long dimensions of sarcocysts were obtained. In those cats with sarcocyst infection, additional fresh muscle tissue was examined with a dissecting microscope at 10 to 40×, and sarcocysts dissected out and placed either in Hank's Balanced Salt Solution (HBSS, Mediatech, Herndon, VA) for molecular characterization or Trump's fixative for transmission electron microscopy (TEM). Photomicrographs were taken of both fixed and fresh specimens. For TEM, samples were transferred to the Electron Microscopy Core Laboratory (Biotechnology Program, University of Florida). There, samples were further processed in 1% osmium tetroxide (w/v) and dehydrated in alcohol prior to embedding in EmBed epoxy resin (Electron Microscopy Sciences Fort Washington, Pa). Seventy to eighty nm thin sections were double stained in 2% aqueous uranyl acetate and Reynolds lead citrate and examined with a Hitachi H-7000 transmission electron microscope (Pleasanton, CA) at 75 keV. Digital photomicrographs were taken with a Gatan Multiscan camera (Pleasanton, CA).

Molecular Characterization of Sarcocysts

A small portion of quadriceps muscle from a cat with no histological evidence of sarcocyst infection or *S. neurona* antibody was used as a negative control for molecular studies. This tissue, and samples of dissected sarcocysts in HBSS, were pelleted individually by centrifugation, then mechanically disrupted by repeatedly pressing the pelleted material against the sides and bottom of the tubes with pipette tips. In each of the tubes, total genomic DNA was purified through the use of a QIAamp® DNA Mini Kit (QIAGEN Inc., Valencia, CA) and supplied protocol. The polymerase chain reaction (PCR) was applied to cat sarcocyst DNA, cat muscle DNA (control), and DNA prepared from culture-derived merozoites of the UFsn-1 *S. neurona* isolate (Ellison et al., 2002,

Long et al., 2002) and *S. falcatula* (ATCC 50701, Manassas, VA). Three PCR reactions were performed. The first utilized the random amplified polymorphic DNA (RAPD)-derived primer pair JNB25/JD396 used by Tanhauser et al. (1999) to amplify and differentiate DNA from select species of *Sarcocystis*. The second reaction was performed with primer pair JD26/JD37 to amplify a region of the small subunit ribosomal RNA (ssurRNA) gene (Dame et al., 1995). The third reaction used JNB69/JNB70 primers to amplify the internal transcribed spacer region 1 (ITS-1) of the rRNA gene (Tanhauser et al., 1999). PCR products were separated by agarose-gel electrophoresis incorporating ethidium bromide and photographed under ultraviolet light.

Amplified ssurRNA gene DNA, corresponding to two separate samples of cat sarcocyst, and one sample of cat muscle (control), were purified prior to sequencing by cutting bands of interest from the agarose gel and utilizing a StrataPrep™ DNA Gel Extraction Kit (Stratagene Inc., La Jolla, CA) and supplied protocol to remove agarose and contaminants. The ssurRNA sequencing primers were the same as those used for amplification.

Two samples of amplified cat sarcocyst DNA from the ITS-1 region were cloned directly into the pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA), and five positive colonies were sequenced with internal, nested primers JNB68/JNB71. ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction kits (PE Biosystems, Foster City, CA) and an Applied Biosystems 377 automated sequencing system (Applied Biosystems, Foster City, CA) were used for sequencing reactions. The BLAST program (Altschul et al., 1997) was used for to search for sequence homologies. Sequence alignments were done using CLUSTALW (version 1.8, Thompson et al., 1994) software and nonweighted

parameters and GCG®s GAP (Genetics Computer Group, Madison, WI) software utilizing end-weighted parameters. An unrooted phylogenetic tree was plotted from aligned ITS-1 sequences with CLUSTALW incorporating the neighbor-joining (N-J) method to illustrate phylogenetic relationships based upon percent divergence (distance) of sequence. Sequence data for cat sarcocyst ssurRNA and two representative clones of ITS-1 DNA were submitted to GenBank™, accession numbers AY190080, AY190081, and AY190082 respectively.

Serologic Testing

A mature SPF cat, born and raised indoors, and fed only processed commercial food, was used to provide control serum for immunoblot tests. Prior to inoculation with killed *S. neurona* merozoites, the cat was anesthetized under isoflurane/O_2 anesthesia, and 50 mL blood collected by jugular venipuncture. Serum was then obtained and frozen at –80 C until further use. Tissue-culture-grown merozoites from the UFsn-1 isolate of *S. neurona*, were harvested from culture flasks and purified by differential centrifugation. Merozoites were then killed by a 30-minute incubation in a 60 C water bath, rinsed in sterile 0.9% saline solution, and counted on a hemacytometer. For each inoculation, 1×10^5 merozoites were suspended in 0.5 mL sterile 0.9% saline and injected intramuscularly. Three inoculations were given, each two weeks apart. Seven days after the 3rd inoculation, 50 mL blood was collected under anesthesia, serum harvested, and stored frozen at –80 C. Pre and postimmunization serum samples were sent to the EPM Diagnostic Laboratory at Neogen Corp, Lexington, KY for immunoblot analysis for antibodies against *S. neurona*.

The immunoblot procedure, typically performed on equine serum, had previously been adapted for use with armadillo serum (Tanhauser et al., 2001). The procedure was further modified for use with cat serum by substituting Protein A/G-peroxidase conjugate in place of Protein G-peroxidase conjugate (both: Pierce, Rockford, IL). Per Neogen Corp., immunoblotting techniques are as follows: Culture-grown *S. neurona* merozoites, originally derived from a horse with EPM, were solubilized and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 4 to 20% tris-glycine gels (Invitrogen, Carlsbad, CA) incorporating Invitrogen Multimark molecular weight standards. Gels were then placed in an electrophoretic transfer device (BioRad, Hercules, CA) for protein transfer to 0.2 μm nitrocellulose membranes (Invitrogen). Membranes were blocked overnight with blotto (phosphate buffered saline, 5% w/v non-fat dried milk, 1% antifoam solution, 0.1% sodium azide) then placed in a miniblotter apparatus (Immunetics, Cambridge, MA). Serum samples, and positive and negative controls, were diluted 1:10 in blotto and added to the template lanes. Following a 90-minute incubation at room temperature, the blot was removed from the template and washed 4 times in phosphate-buffered saline containing 0.1% Tween-20 (PBST). Protein A/G-peroxidase conjugate, diluted in PBST, is added to the blot for 60 min then washed off, and the blot developed by the addition of 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate (Vector Laboratories, Burlingame, CA). Developed blots were dried, interpreted by examination, and in selected cases, scanned with a BioRad GS-700 imaging densitometer to quantify results.

Serum samples collected from 50 cats examined for sarcocysts, 50 feral cats from trap-neuter-return programs, and control cat sera were assayed. Negative controls

included preimmune cat serum and *S. neurona*-negative horse serum. Immune cat serum and horse serum, both strongly and weakly reacting, were used as positive controls.

In order to learn if sarcocyst infection might be linked to immunosuppression, cat sera were tested for immunosuppressive retroviral infections. A commercially available kit (SNAP® FIV Antibody/FeLV Antigen Combo Test kit, IDEXX Laboratories, Portland, ME) was used to test all serum samples for feline leukemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibody.

Opossum Challenge

A laboratory-raised opossum, fed only a commercial laboratory diet since weaning, was fed fresh muscle from five cats infected with sarcocysts. Prior to feeding cat muscle, opossum feces were examined microscopically for the presence of sporocysts using the Sheather's sugar flotation technique. Muscle from each of the five cats was chopped, mixed together with commercial, canned cat food and fed daily for four days. Approximately 30 g of muscle were fed in total. Seven days after the last feeding of cat muscle, and three times weekly thereafter, opossum feces were checked for sporocysts. Thirty days after the last feeding of cat muscle, the opossum was euthanized with an overdose of sodium pentobarbital (Euthasol, Delmarva Laboratories, Midlothian, VA). The small and large intestines were removed and split along their length with sterile scissors. A glass microscope slide was used to scrape epithelia and intestinal contents from the intestinal lumen. Scrapings were collected into a sterile blender cup and homogenized for 1 minute. The homogenate (approximately 35 mL) was transferred to a sterile 500 mL flask, 400 mL of deionized water was added, and the mixture stirred for 4 hours. A portion of the mixture was strained through a tea strainer into two centrifuge

tubes. Tubes were centrifuged at 2330 × g for 10 minutes, and the supernatant decanted. Tubes were filled with Sheathers sugar solution (specific gravity 1.27), and a coverslip applied to the top of the tubes. Tubes were centrifuged at 2330 × g for 10 minutes. Coverslips were then applied face down onto glass slides and examined at 100× for the presence of sporocysts.

Bradyzoite Culture

Samples of sarcocysts from each of the cats infected with sarcocysts were dissected from muscle and each placed in 0.5 mL Hank's Balanced Salt Solution (HBSS) containing 200 U penicillin, 0.2 mg streptomycin, and 0.5 μg amphotericin B (Antibiotic Antimycotic Solution, Sigma, St. Louis, MO) per mL. Samples were rinsed twice in this solution by centifugation at 5000 × g. After the final rinse, a sterile pipette tip was used to rupture sarcocysts and release bradyzoites. Bradyzoite suspensions were transferred to individual 25 cm^2 flasks containing 75% confluent monolayers of bovine turbinate (BT 1390, ATCC CRL 1390) cells in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Ft. Collins, CO), and 10 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin and 0.1 mg streptomycin (each, Mediatech, Herndon, VA) per mL. Twice weekly, two thirds of the media was aspirated and replaced with fresh media. At 16 and 32 weeks postinfection, cultures were trypsinized with 2mL 0.05% trypsin-EDTA solution (Mediatech, Herndon, VA) and one third of the cells transferred to a new 25cm^2 flask. Cultures were monitored microscopically at 100 and 200× for evidence of monolayer infection. Cultures were monitored for 60 weeks postinfection.

Molecular Analysis of Fixed Tissue from Florida Panther Containing Sarcocysts

Hematoxylin and eosin stained sections from a 1988 – 1989 study by Greiner and colleagues (Greiner et al., 1989) of muscle sarcocysts in Florida panthers were re-examined microscopically at 40 – 100× for the presence of sarcocysts. The formalin-fixed, paraffin-embedded tissue block for panther # 07 tongue was chosen for molecular analysis as microscopic examination of sections obtained from this specimen showed this it to contain more sarcocysts (approximately 10) than other specimens examined.

DNA extraction from formalin-fixed, paraffin-embedded tongue was performed. Eight samples were prepared. For each sample, five slices were shaved from the tissue block, totaling approximately 50 mg, and placed in a microcentrifuge tube with 800 µl of xylene. The tube was vortexed for five seconds, and 400 µl ethanol then added. The tube was again vortexed for five seconds, then centrifuged five minutes at 10,000 × g, and the supernatant decanted. This de-paraffinization process was repeated twice. After the final rinse, the tube was inverted and allowed to dry for 20 minutes before 250 µl of lysis buffer (1% sodium dodecyl sulfate, 100 mM EDTA, 50 mM Tris-HCl, 100 mM NaCl) were added to the tube, along with 62.5 µg of proteinase K (Research Products International, Mt. Prospect, IL) and incubated overnight at 55C. The following day, an additional 62.5 µg of proteinase K were added and incubated one hour at 65C. After cooling to room temperature, and equal volume (375 µl) of 4M ammonium acetate was added and the tube vortexed. Twice the volume (750 µl) of isopropyl alcohol was then added to the tube to precipitate the DNA. The mixture was cooled to 4C and centrifuged 10 minutes at 14,000 × g. The supernatant was decanted and pellet rinsed twice by

centrifugation at 14,000 × g with 800 µl of cold (-20C) ethanol. After the final rinse, the supernatant was decanted and the tube inverted over absorbent paper to dry the pellet. The pellet was resuspended in 20 µl of molecular grade water (Invitrogen, Carlsbad, CA) and allowed to dissolve overnight. Prior to analysis by PCR, the solution was pooled to form two aliquots and each centrifuged five minutes at 10, 000 × g. Supernatant from one of the tubes was further purified by standard phenol/chloroform extraction followed by 3M sodium acetate/cold ethanol precipitation of DNA.

PCR with primer pair JD26/JD37 was used for amplification of a region of the ssurRNA gene (Dame et al., 1995). DNA prepared from culture-derived merozoites of the UFsn-1 *S. neurona* isolate (Ellison et al., 2002, Long et al., 2002) and *S. falcatula* (ATCC 50701, Manassas, VA) were used as positive controls, and water as negative control. PCR products were separated by low-melt agarose-gel (SeaPlaque GTC, Biowhittaker Molecular Applications, Rockland, ME) electrophoresis incorporating ethidium bromide and photographed under ultraviolet light. The resultant ssurRNA gene amplicon was cut from the gel, melted, and purified prior to sequencing with a QiaQuick™ PCR Kit (Valencia, CA) and supplied protocol. ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Foster City, CA) and an Applied Biosystems 377 automated sequencing system (Applied Biosystems, Foster City, CA) were used for sequencing reactions. The ssurRNA sequencing primers were the same as those used for amplification. The BLAST program (Altschul et al., 1997) was used for to search for sequence homologies.

CHAPTER 4
RESULTS

Histologic examination of muscle samples revealed sarcocysts in 5 of 50 cats (10%). Sarcocysts were most abundant in sections of quadriceps muscle. No inflammatory reaction was seen in tissue surrounding the sarcocysts. In some instances, sarcocysts were visible grossly in fresh muscle. Sarcocysts appeared filamentous, sometimes convoluted, and measured approximately 0.1 × 0.5 - 20 mm (width × length) (Figs. 4-1 and 4-2). Bradyzoites from fresh sarcocysts measured 3.3 × 8.8 µm (width × length) (Fig. 4-3). Additional photomicrographs from light microscopy are shown in Figures 4-4 and 4-5.

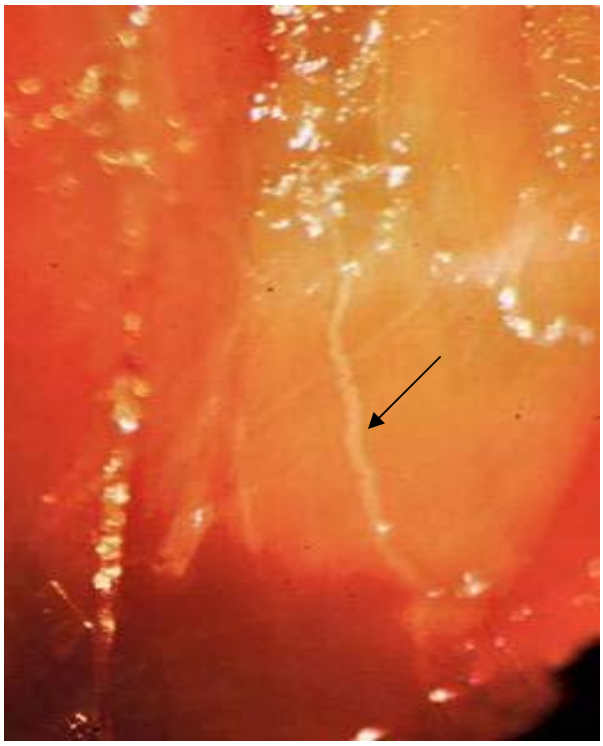


Figure 4-1. Photograph of sarcocyst (arrow) in cat skeletal muscle, 16×. Some sarcocysts were visible grossly and measured 0.1 × 0.5 to 20 mm (width × length).

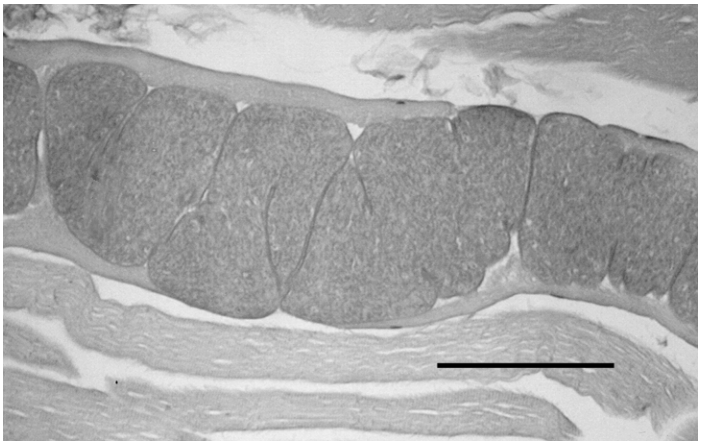
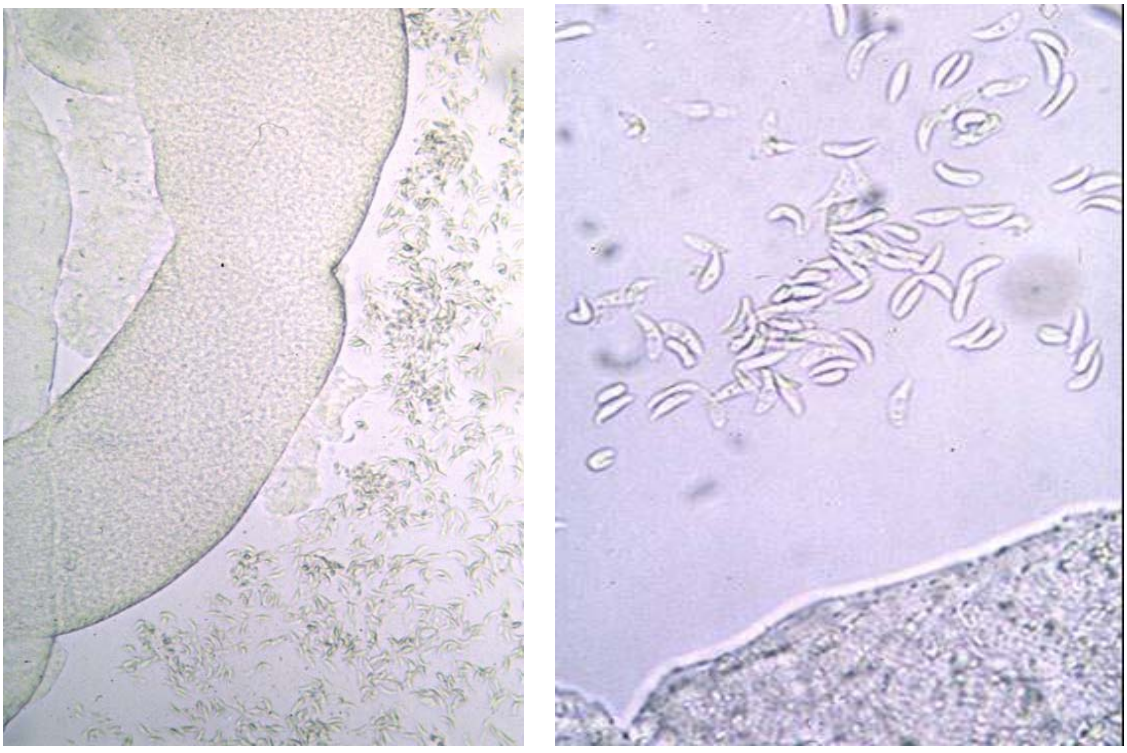
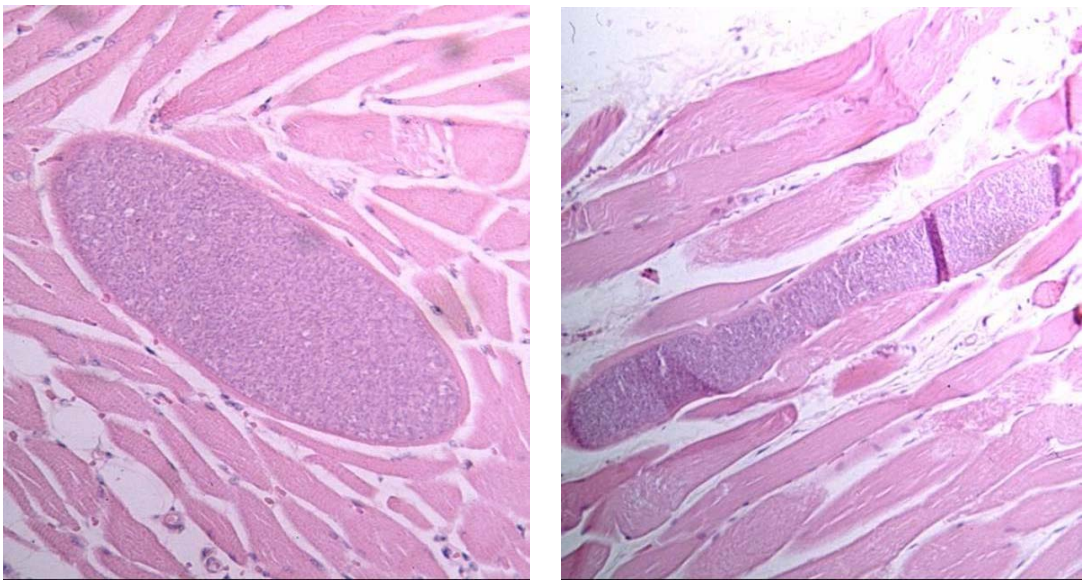


Figure 4-2. Portion of sarcocyst located in quadriceps muscle of cat. Hematoxylin and eosin stain. Scale bar =100 μm.



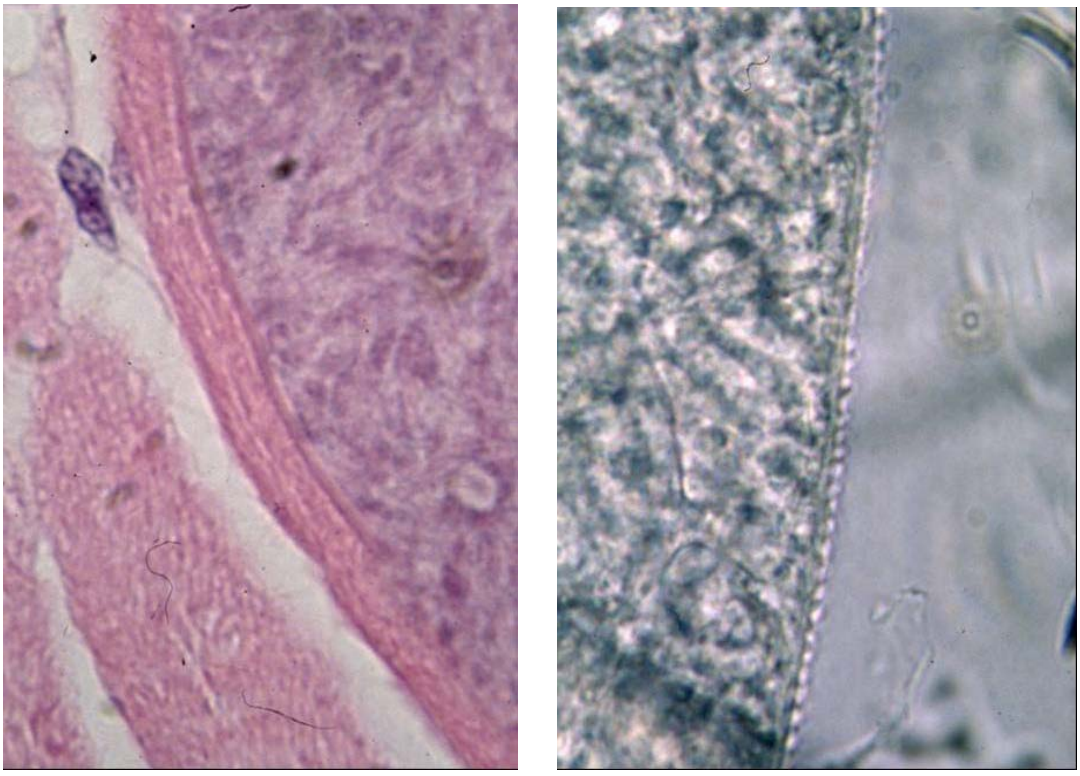
A B

Figure 4-3. Photomicrographs from light microscopy of fresh sarcocysts obtained from cat muscle. A) convoluted, "wavy" shaped sarcocyst filled with bradyzoites and surrounded by bradyzoites that have spilled out of the ruptured sarcocyst, 160×. B) close up of same (400×); fresh bradyzoites measure 8.8 × 3.3 μm.



A B

Figure 4-4. Photographs taken from light microscopy of H & E stained sections of cat skeletal muscle containing sarcocysts. A) 297 × 113 μm (160×). B) 500 × 160 μm (100×).



A B

Figure 4-5. Photomicrographs from light microscopy of the sarcocyst wall of A) fixed, H & E stained and B) fresh sarcocysts obtained from cat muscle, 1000×.

Images from TEM (Figs. 4-6 and 4-7) show a thin (maximal thickness approximately 2.5 μm), relatively simple, sarcocyst wall with short, irregular, villar protrusions with rounded tips. The electron-dense layer underlying the parasitophorous vacuolar membrane was interrupted at intervals, especially in areas at the base of, or between, villi. The ground substance was devoid of microtubules, even as it formed villar protrusions, and was continuous with the septa that run between bradyzoites and metrocytes. Bradyzoites measured approximately 6 - 8 × 1.5 - 3 μm. The anterior region of the bradyzoites contained numerous micronemes. Inclusion bodies, including amylopectin, lipid, and electron-dense granules, were found in the center region of the bradyzoites, and cell organelles, including the nucleus, were confined primarily to the posterior regions. Sarcocyst morphology was compared to published descriptions of sarcocysts found previously in both domestic cats and wild felid species. Based upon these comparisons, it was determined that sarcocysts found in this study were *Sarcocystis felis*.

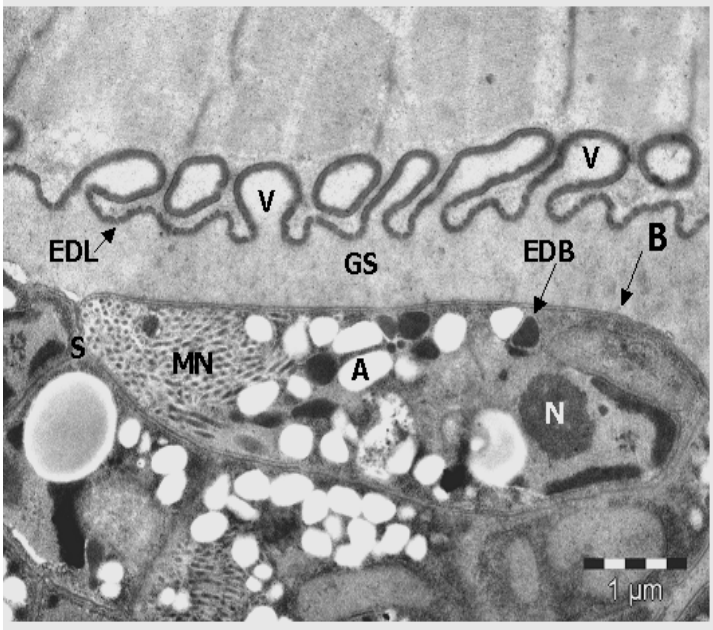


Figure 4-6. Photomicrograph from TEM of cat sarcocyst. Sarcocyst wall shows interruptions of the electron-dense layer (EDL, arrow) underlying the parasitophorous vacuolar membrane, particularly at the base of, and between, villi (V). Villi and ground substance (GS) lack microtubules. Bradyzoites (B, arrow) contain micronemes (MN), amylopectin granules (A), electron-dense bodies (EDB, arrow), and nucleus (N). Septa (S) separate bradyzoites within the sarcocyst.

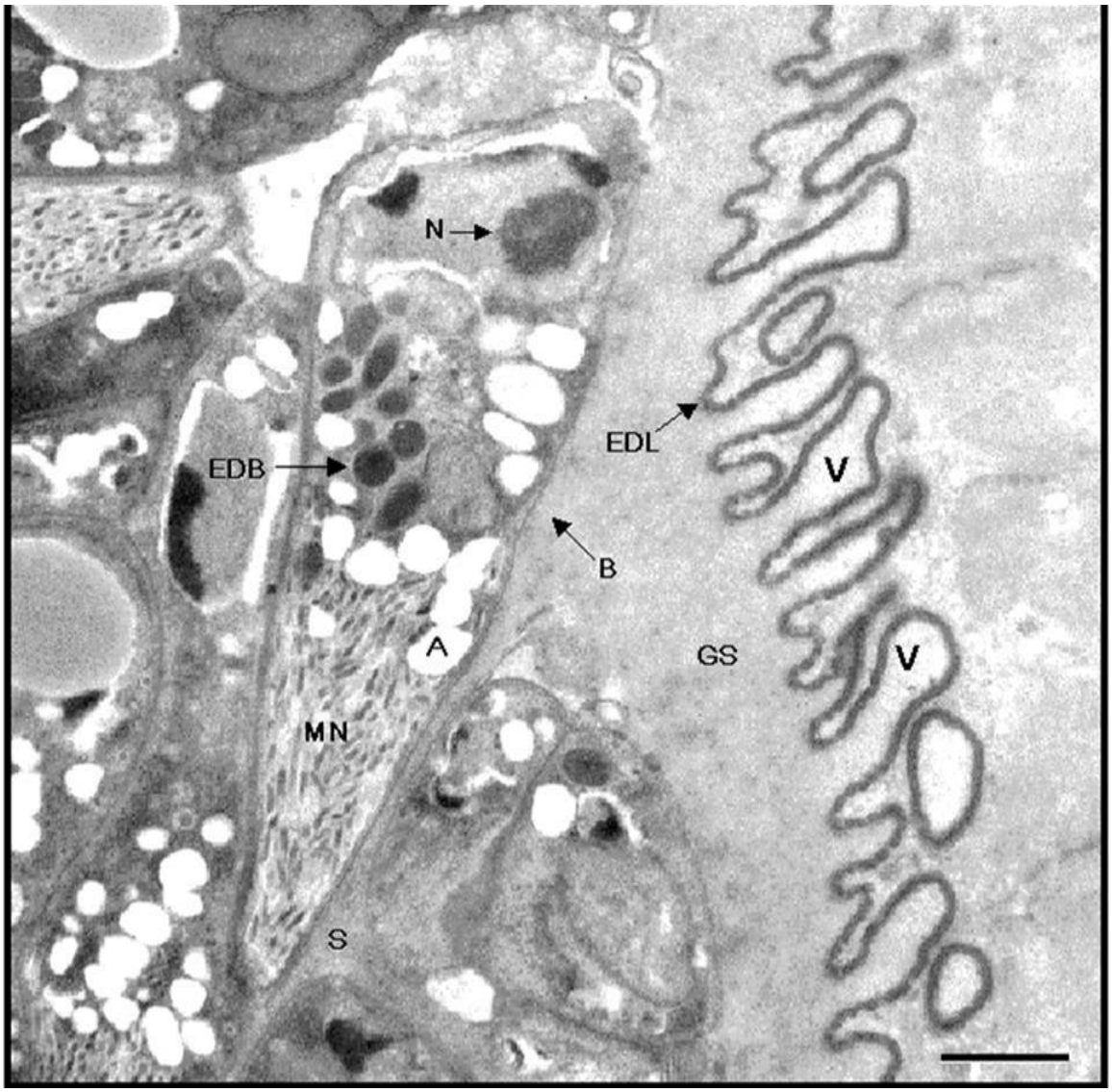


Figure 4-7. Photomicrograph from TEM of cat sarcocyst. Villi (V) have rounded tips and contain no microtubules. "Hobnailed" appearance of electron dense layer (EDL) is evident. Ground substance (GS) and a bradyzoite (B, arrow) are also shown. Bradyzoite contains amylopectin granules (A), electron dense bodies (EDB) and micronemes (MN). Scale bar = 1 μm.

Amplification of cat sarcocyst DNA or cat muscle DNA was not seen when PCR was performed with RAPD-derived primer pair JNB25/JD396. PCR using JD26/JD37 (ssurRNA) primers yielded single PCR products of approximately 410 and 450 bp from cat sarcocyst, and cat muscle samples, respectively (Fig. 4-8).

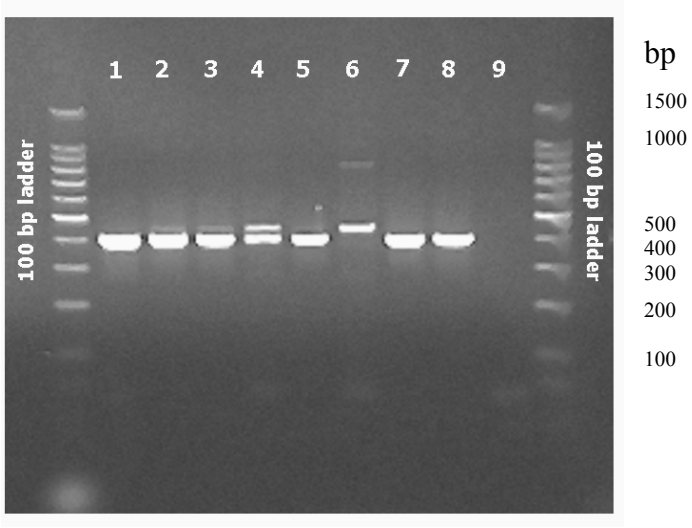


Figure 4-8. Agarose-gel electrophoresis showing results of PCR using ssruRNA gene primer pair JD26/JD37 and template DNA. Lanes 1-5, sarcocyst DNA from cats 1-5 (~ 410 bp). Lane 6, DNA from uninfected cat muscle (~ 450 bp). Lane 7, *S. neurona* DNA (~ 410 bp). Lane 8, *S. falcatula* DNA (~ 410 bp). Lane 9, water control. Fragment size ladder (100 bp) included on both sides of gel; ladder legend (bp) shown at right of photograph. Additional band above primary band in lane 4 (cat 4) most likely indicates muscle contamination of sarcocyst DNA.

JNB69/JNB70 (ITS-1) amplified an approximately 1080-bp product from cat sarcocysts and an approximately 1200-bp product from both *S. neurona* and *S. falcatula* DNA prepared from culture-derived merozoites. Uninfected muscle was not amplified (Fig. 4-9). PCR results were consistent for each of the five samples of cat sarcocyst.

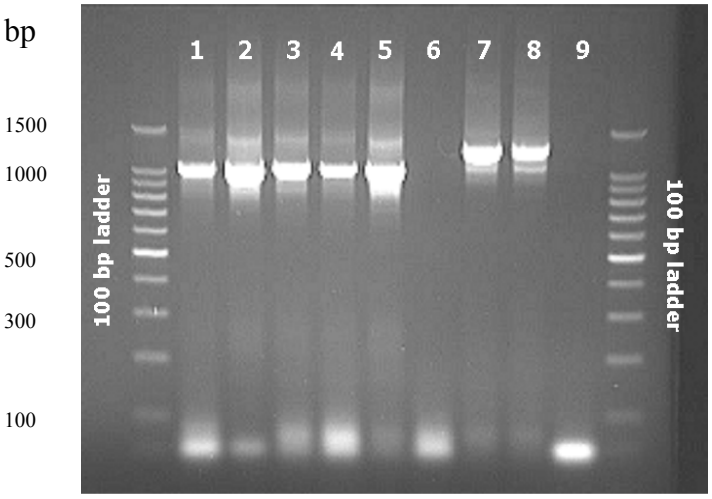


Figure 4-9. Agarose-gel electrophoresis showing results of PCR using ITS-1 primer pair JNB69/JNB70 and template DNA. Lanes 1-5, sarcocyst DNA from cats 1-5 (~ 1080 bp). Lane 6, DNA from uninfected cat muscle. Lane 7, *S. neurona* DNA (~ 1200 bp). Lane 8, *S. falcatula* DNA (~ 1200 bp). Lane 9, water control. Fragment size ladder (100 bp) included on both sides of gel; ladder legend (bp) shown left of photograph.

Sequence data from the ssurRNA gene of cat sarcocysts showed significant identity with archived ssurRNA sequence from many *Eimeriidea* organisms, particularly *S. neurona* (GenBank U33149) and *S. falcatula* (GenBank U35075). By CLUSTALW and GAP, both of these organisms have a 99% identity with cat sarcocyst ssurRNA sequence. Archived ssurRNA sequence was available for 6 of the reported 15 species of *Sarcocystis* that use a felid definitive host. When compared to ssurRNA sequence generated from cat sarcocysts, the following identity-based, global alignment scores were obtained from CLUSTALW for these definitive host species: S. *muris* 98% (GenBank M64244), *S. hirsuta* 92% (GenBank AF017122), *S. buffalonis* 92% (GenBank AF017121), *S. fusiformis* 92% (GenBank U03071), *S. gigantea* 91% (GenBank L24384), and *S. rodentifelis* 82% (GenBank AY015111). After accounting for priming losses and terminal sequences of 18srRNA (40 bp) and 5.8srRNA (30 bp) DNA, approximately 800 bp of ITS-1 sequence was generated from each of the five cat sarcocyst DNA clones. Alignment of these cloned ITS-1 sequences showed limited base switching among clones; by GAP, there was 98% identity of sequence among clones. Cloned cat sarcocyst DNA from the ITS-1 region showed only 45% identity with *S. neurona* (UCD1 isolate, GenBank AF081944) and 46% with *S. falcatula* (Florida 1 isolate, GenBank AF098244) sequence when aligned with GAP. Pairwise, global alignment scores from CLUSTALW for ITS-1 region sequence of cat sarcocyst were only 6% for *S. neurona* (UCD1 isolate, GenBank AF081944), 11% for *S. falcatula* (Florida 1 isolate, GenBank AF098244), 4% for *Hammondia heydorni* (CZ-3 isolate, GenBank AF317281), 12% for *Toxoplasma gondii* (ME49 isolate, GenBank L49390), and 5% for *Neospora caninum* (Liverpool isolate, GenBank L49389). An unrooted, phylogenetic, N-J tree (Fig. 4-10), shows the

evolutionary relationship of ITS-1 sequence from these organisms and that from cat sarcocyst.

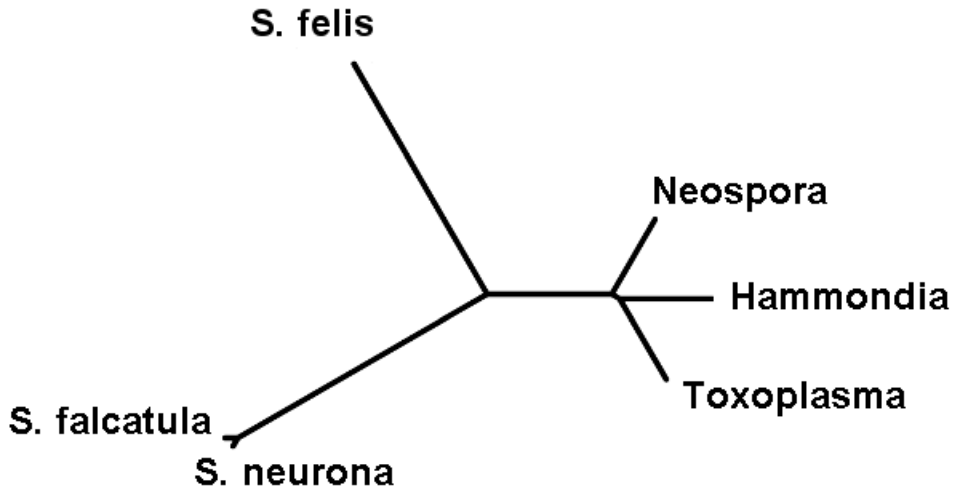


Figure 4-10. Unrooted phylogenetic tree showing the divergence of *Sarcocystis felis* ITS-1 gene sequence to that from *Sarcocystis neurona*, *Sarcocystis falcatula*, and organisms from related genera: *Toxoplasma gondii*, *Hammondia heydorni*, and *Neospora caninum*. Tree was plotted from CLUSTALW, incorporating the neighbor-joining (N-J) algorithm.

Inoculation of a cat with the UFsn-1 isolate of *S. neurona* resulted in seroconversion against 17-kDa and 30-kDa proteins of *S. neurona*, shown by immunoblot (Fig. 4-11). The 17-kDa band had the same apparent molecular mass as an *S. neurona*-specific band recognized by equine serum. Pre-immunization cat serum was negative for reactivity with the 17-kDa antigen. Of the 50 serum samples collected from cats examined for sarcocysts, 2 samples (4%) were positive by immunoblot for antibody to *S. neurona*. One of five cats harboring sarcocysts was seropositive by immunoblot. Immunoblot testing of the 50 sera collected from feral cats brought to trap-neuter-return clinics resulted in 3 positives (6%). Combined, 5/100 (5%) cat sera evaluated in this study were seroreactive with *S. neurona.*

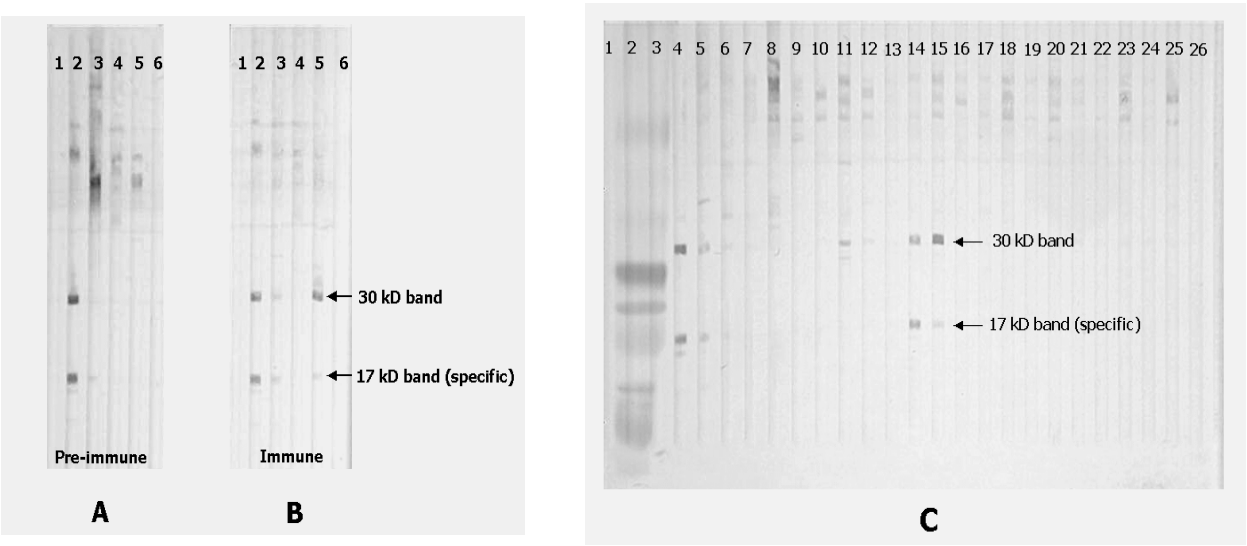


Figure 4-11. Immunoblots of cat serum. A) Blot of pre-immune cat serum. Lane 1: no sample; blotto only. Lane 2: strongly positive horse serum from horse infected with *S. neurona*. Lane 3: weakly positive horse serum from horse infected with *S. neurona*. Lane 4: horse serum from non-infected animal. Lane 5: serum from cat prior to immunization with *S. neurona*. Lane 6: no sample, blotto only. B) Blot of post-immunisation cat serum. Lane 1: no sample; blotto only. Lane 2: strongly positive horse serum from horse infected with *S. neurona*. Lane 3: weakly positive horse serum from horse infected with *S. neurona*. Lane 4: horse serum from non-infected animal. Lane 5: serum from cat post-immunization with the UFsn-1 isolate of *S. neurona*. Lane 6: no sample; blotto only. C**)** Molecular weight standards overlay lanes 1-3, which contain blotto solution only. Lane 4: strongly positive horse serum from horse infected with *S. neurona*. Lane 5: weakly positive horse serum from horse infected with *S. neurona*. Lane 6: horse serum from non-infected animal. Lanes 7-25: serum from feral cats. Lane 26 contains blotto solution only. Cat serum added to lanes 14 and 15 is considered positive for antibody to *S. neurona*.

FeLV antigen and FIV antibody testing of serum collected from the 50 cats euthanized at the local shelter resulted in 2 cats positive for FeLV antigen (4%), and 2 different cats positive for FIV antibody (4%). One cat positive for FeLV antigen had sarcocyst infection. No FIV antibody-positive cats had sarcocysts. No FeLV or FIV-positive cats from this group were positive for antibody to *S. neurona*. Testing of the serum from the 50 additional feral cats yielded 2 cats (4%) positive for FeLV antigen and

a different cat (2%) positive for FIV antibody. One cat positive for FeLV antigen was also positive for antibody to *S. neurona*. Therefore, of the 100 cats evaluated in this study, 4 were positive for FeLV antigen (4%), and 3 for FIV antibody (3%). Results are shown in Tables 4-1 and 4-2 below.

Table 4-1. Results of serology and histology for 9 of 50 cats euthanized at the local animal shelter

Cat ID	*S. neurona* Specific Ab	FeLV Ag	FIV Ab	Sarcocysts	Sex / Status	Source
56710	Pos	Neg	Neg	Pos	F, pregnant	Stray
57559	Neg	Neg	Neg	Pos	M, intact	Stray
57605	Neg	Neg	Neg	Pos	M, intact	Stray
58004	Neg	Pos	Neg	Pos	M, intact	Stray
57270	Neg	Neg	Neg	Pos	M, intact	Stray
56709	Pos	Neg	Neg	Neg	M, intact	Stray
57248	Neg	Pos	Neg	Neg	F, intact	Stray
57561	Neg	Neg	Pos	Neg	M, intact	Stray
57601	Neg	Neg	Pos	Neg	M, intact	Stray

Data for cats not shown was negative.

Table 4-2. Results of serology for 8 of 50 cats taken to trap-neuter-return clinics

Cat ID	*S. neurona* Specific Ab	FeLV Ag	FIV Ab	Sex	Source
F0-455	Pos	Pos	Neg	M	Trapped
F0-514	Pos	Neg	Neg	M	Trapped
F0-505	Pos	Neg	Neg	F	Trapped
F0-564	Neg	Pos	Neg	F	Trapped
F9-1519	Neg	Neg	Pos	M	Trapped
F0-192	Neg	Neg	Neg	F	Trapped
F0-205	Neg	Neg	Neg	F	Trapped
F9-1416	Neg	Neg	Neg	M	Trapped

Data for cats not shown was negative.

Sporocysts were not found in opossum feces that were collected after feeding sarcocyst-infected cat muscle, and sporocysts were not found in intestinal mucosal scrapings from the opossum.

No evidence of infection was noted in bovine turbinate cell cultures inoculated with bradyzoites. Cultures were discarded 60 weeks post-inoculation.

PCR of DNA isolated from formalin-fixed Florida panther tongue yielded a faint band of approximately 410 bp. Positive control DNA yielded bands of equivalent sizes (Fig. 4-12).

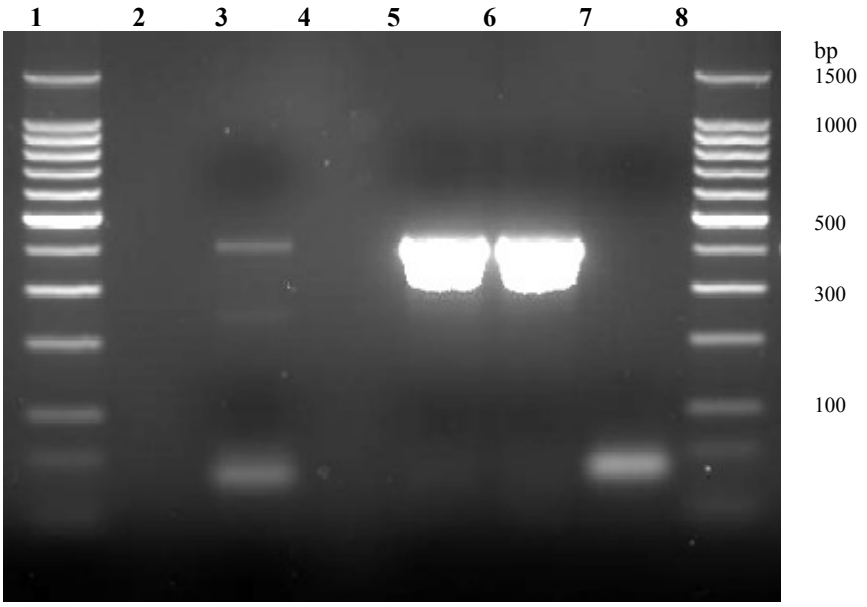


Figure 4-12. Agarose-gel electrophoresis showing results of PCR using ssruRNA gene primer pair JD26/JD37 and DNA prepared from formalin-fixed, paraffin-embedded Florida panther tongue containing sarcocysts. Lane 3, product amplified from formalin-fixed, paraffin-embedded Florida panther tongue containing sarcocysts (~ 410 bp). Lane 5, *S. neurona* DNA (~ 410 bp). Lane 6, *S. falcatula* DNA (~ 410 bp). Lane 7, water control. Lanes 2 and 4 are empty, done to minimize DNA contamination when cutting band from lane 3. Fragment size ladder (100 bp) included on both sides of gel in lanes 1 and 8; ladder legend (bp) given at right of photograph.

Approximately 360 nucleotides of sequence were generated and submitted for BLASTn analysis. Sequence homology data from BLAST found the product amplified from the Florida panther tissue locally homologous with ssurRNA sequence from plant

material, most notably (89%) from the genus *Pinus* (pine). No homologies to other *Sarcocystis* or protozoal organisms were found.

CHAPTER 5
DISCUSSION

Because of the importance of *S. neurona* as an equine pathogen, there has been an active effort to identify relevant hosts for this organism. As a result of the work described here, it was discovered that sarcocysts were fairly common in cats; however, on the basis of careful morphological, molecular, and biological characterization, these sarcocysts could not be classified as *S. neurona*, or even *S. neurona*-like.

Our study is the largest reported survey for sarcocysts in domestic cats; five of 50 (10%) cats had sarcocysts. Previously, sarcocysts were found in four of 12 cats in Indiana (Everitt et al., 1987) and one of nine cats in Missouri (Turay et al., 2002). In another study, sarcocysts were collected from muscle biopsies of five cats, however no prevalence data were provided (Fiori et al., 1988). Higher prevalence of sarcocysts has been reported from surveys of wild felid species. In Florida, 50% of bobcats (Anderson et al., 1992), and 83% of free-ranging Florida panthers and cougars (Greiner et al., 1989) were infected. Sixty-six percent of Arkansas bobcats (Dubey et al., 1992) and 70% of captive born and raised cheetahs (Briggs et al., 1993) had sarcocysts. Sarcocysts were found in 100% of adult, wild lions from Namibia, but not in juveniles, suggesting that infection increases with maturity (Kinsel et al., 1998). Sarcocysts are prevalent in Felidae and appear to have a worldwide distribution.

The population of cats we examined for sarcocysts included (few) surrendered pet cats, free-roaming cats, socialized strays, and feral cats and thus may be expected to have a sarcocyst prevalence somewhere between that found in wild felids and that found in

domestic cats kept indoors. In the wild, increased opportunities exist for cats to ingest sporocysts from definitive host(s); environment and diet no doubt contribute to the increased prevalence of sarcocyst infection in wild species. Captive wild species are typically fed raw meat and offal, potentially exposing them to viable sporocysts. Although the cats in the present study received unknown levels of human care, one may assume some had access to processed foods, shelter, and preventative health care for at least part of their lives; factors that may explain the comparatively low prevalence of sarcocysts found in domestic cats. Data supplied by the local shelter indicated all five infected cats were listed as "strays", evidence consistent with the notion that stray or feral cats may be more likely to be infected.

General health and immunity may also be factors for sarcocyst infection. Anderson and colleagues (1992) could not conclude that the high rate of sarcocyst infection among bobcats was linked to specific viral immunosuppressive diseases, however, Greiner et al. (1989) found that 78% of Florida panthers were seropositive for feline panleukopenia virus, not an immunosuppressive virus on its own, but one that might contribute to poor health (Dr. Julie Levy, University of Florida, personal communication) and 30% were positive for FIV. Both Florida panthers and captive populations of cheetahs are reported to be inbred and to lack genetic diversity, possibly contributing to poor immunity. In a survey of FeLV and FIV infection in 1143 free-roaming domestic cats sampled from the same geographical area as those from this study, incidence of FeLV antigen and feline FIV antibodies was 3.7% and 4.3%, respectively (Lee et al. 2002). In our study, the apparent prevalence of FeLV was 4% and FIV 3%. Feline leukemia virus antigen was detected in one of five cats positive for sarcocysts and one different cat positive for

antibody to *S. neurona*; however, the low prevalence of these potentially immunosuppressive retroviral infections makes it difficult to draw a conclusion regarding any association with *Sarcocystis* infection.

In both domestic cats and wild felid species, sarcocyst morphology has been described incidental to examination of muscle tissue collected at necropsy from debilitated and healthy animals. In these reports, histologic examination revealed sarcocysts of varying sizes: from 24 μm (Kirkpatrick et al., 1986) to 270 μm (Everitt et al., 1987) in diameter by 24 μm (Everitt et al., 1987) to 2100 μm in length (Dubey et al., 1992). The ultrastructure of these sarcocysts was virtually identical in all cases: rounded, irregularly-spaced villi devoid of microtubules, a regularly interrupted electron-dense layer underlying the parasitophorous membrane, and septae separating bradyzoites. Bradyoites measured approximately 10 μm long. It was noted that ultrastructural morphology was not consistent with descriptions of sarcocysts for which the cat is known to serve as a definitive host (Kirkpatrick et al., 1986). Citing the morphological similarity illustrated for felid sarcocysts, Dubey and colleagues (1992) formally identified them as *Sarcocystis felis*.

Based upon a descriptive classification of *Sarcocystis* species (Dubey et al., 1989), the sarcocyst wall of *S. felis* has been classified as a type 4 (Odening, 1998) or a type 9 without villar microtubules (Dubey et al., 1992). The ultrastructural morphology of *S. neurona* sarcocysts has been described for infections in cats (Dubey et al., 2000, Dubey et al., 2001c), armadillos (Cheadle et al., 2001a), raccoons (Dubey et al., 2001a), and skunks (Cheadle et al., 2001b). Sarcocysts were less than 200 μm in length; the sarcocyst wall was comprised of long, slender villi containing microtubules, consistent

with a classification of type 11 or 12 (Dubey et al., 1989). Bradyzoites were also slender and 5-7 μm long. In another report, sarcocysts induced in cats inoculated with *S. neurona* or *S. neurona*-like merozoites were examined by light microscopy only; photomicrographs show sarcocysts measuring 20 μm x 140 μm (Butcher et al. 2002). Photomicrographs from TEM of the sarcocyst wall of *S. neurona* obtained from skunk muscle (Cheadle et al., 2001b) are very different than photomicrographs of TEM from cat sarcocysts shown in Figs. 4-6 and 4-7.

Sarcocysts seen in the present study are different than *S. neurona*, but closely resemble *S. felis*. Both histologic sections and fresh tissue were examined (Figs. 4-1 through 4-5). Only one previous report (Greiner et al., 1989), describes sarcocysts in fresh tissue (muscle squash preparations) and reports their length to be comparable (1 cm) to some found in this study. In fresh tissue, some sarcocysts seen in this study were large enough to be visible grossly, up to 2 cm in length, and many appeared to have a convoluted or wavy shape.

Compared to other *S. neurona* intermediate hosts, domestic cats, as companion animals, are under close observation by their owners and sent in great numbers to veterinarians for evaluation of health problems, yet few reports in the veterinary literature describe sarcocystosis, disease associated with widespread sarcocyst infection, or *Sarcocystis*-associated neurological disorders in domestic cats. Just one reference describes encephalitis in domestic cats caused by infection with a *Sarcocystis* spp., (Dubey et al. 1994). A second citation (Dubey and Hamir 2000), further characterizes the organism found, and identifies it as *S. neurona*. In experiments designed to demonstrate and confirm the domestic cat as an intermediate host for *S. neurona*, cat subjects, even

when dosed with corticosteroids for immune suppression, did not become diseased (Dubey et al. 2000, Butcher et al. 2002, Turay et al. 2002, Dubey et al. 2002). However, in similar experiments, both skunks (Cheadle et al. 2001) and raccoons (Dubey et al. 2001a) became diseased when dosed with *S. neurona* sporocysts. Naturally-occurring encephalitic infections with *S. neurona* have been documented in raccoons, skunks, mink, and sea otter (reviewed in Dubey et al. 2001d). Considering the comparatively huge numbers of domestic cats, one might expect a greater incidence of morbidity and mortality associated with *S. neurona* infection if cats are indeed an important natural intermediate host for this parasite.

The primer pair JNB25/JD396 does not amplify DNA from all *Sarcocystis* spp. (Tanhauser et al., 2001), but readily amplifies both *S. neurona* and *S. falcatula*. Lack of amplification of cat sarcocyst DNA by these primers suggests the sarcocysts found in cat muscle do not belong to either of these two species. Comparisons among conserved ssurRNA gene sequence did not readily differentiate cat sarcocyst (*S. felis*) DNA found in this study from several other *Sarcocystis* species, including *S. neurona*. However, *S. felis* ssurRNA gene sequence was not similar to that for *Sarcocystis* species that use the cat as definitive host, suggesting sarcocysts seen were not due to an aberrant infection with these definitive host species.

Based upon a local alignment of 316 nucleotides, ITS-1 sequence analysis was used to support the conclusion that raccoons are natural intermediate hosts of *S. neurona* (Dubey et al., 2001a). Although clearly belonging to *Sarcocystis,* and 99% similar to *S. neurona* ssurDNA, *S. felis* sarcocyst DNA sequence was quite dissimilar over the ITS-1 region compared to *S. neurona* and *S. falcatula*. Internal transcribed spacer

region 1 primers amplified a smaller-sized product from *S. felis* sarcocysts than from cultured *S. neurona* or *S. falcatula* merozoites; restriction endonuclease digest of PCR products, typically done to differentiate species at this locus by PCR alone (Tanhauser et al. 1999, Turay et al. 2002), was therefore unnecessary. Local alignments of ITS-1 sequence from cat sarcocyst against archived ITS-1 sequences was done by BLAST. Alignment with many *Eimeriidea* organisms, most notably *S. falcatula* (AF389339) and *S. lindsayi* (AF387164) occurred only in conserved regions of approximately 40 nucleotides (nt) from the 3' end of the 18srRNA gene and 30 nt from the 5' end of the 5.8S gene. Sequence data for the ITS-1 region of the *S. felis* was completely non-homologous with that from *S. falcatula* or two isolates of *S. neurona* when conserved regions were trimmed from the sequence. Although referred to by Marsh et al. 1999, ITS-1 sequence for *S. gigantea* and *S. muris*, definitive host species of the cat, are not available within the public domain for comparison. The divergence of ITS-1 sequence from *S. felis* sarcocysts to those from related coccidian species, even those of the same genus, is evident in the phylogenetic tree (Fig. 4-10).

Once *S. felis* was identified as the source of infection found in cats from this study, an attempt was made to compare ssurRNA genes of sarcocysts in these cats to those from sarcocysts found previously in Florida panthers. Sarcocysts found in Florida panthers (Greiner et al., 1989) were assumed to be *S. felis* as they were morphologically identical to *S. felis* found in bobcats (Dubey et al., 1992). Comparable or identical ssurRNA sequence between panther and domestic cat sarcocysts would have augmented the findings of the present study. Amplification of DNA from formalin-fixed, paraffin-embedded tissue, particularly if it is aged, is more difficult than from fresh

tissue. Moreover, degradation of DNA into smaller fragments frequently occurs (Wickham et al., 2001). Primers JD26/JD37 amplify 410 bp products from both *S. neurona* and *S. falcatula*. It was hoped that this relatively short sequence could be amplified from the sarcocysts located in the panther tongue; there was less optimism for amplifying the larger ITS-1 region, 1080 bp from *S. felis*. Although primers JD26/JD37 did amplify a 410 bp product from the panther tissue (Fig. 4-12), JD26/JD37 are "universal primers", able to amplify ssurRNA from all eukaryotic organisms. The finding of sequence homology to plant material (most significantly with *Pinus*, pine) likely indicates contamination of the sample at some point prior to sequencing. If comparable sequence from JD26/JD37 to *Sarcocystis* had been found, one would still need further analyses to diagnose *S. felis*; the ssurRNA gene amplified by JD26/JD37 cannot differentiate between all *Sarcocystis* species (Tanhauser et al., 1999). To pursue corroborating evidence of *S. felis* in both domestic and previously defined wild felid hosts, two tactics might be employed: obtain fresh samples from these wild species for analysis, or re-design primers for the ITS-1 region that amplify shorter, but diagnostic sections of this gene.

The opossum consumed 30 g of cat muscle containing numerous sarcocysts, yet no sporocysts were found in feces or gut scrapings. Other researchers have fed greater quantities of cat muscle to opossums for bioassay purposes (Dubey et al., 2000, Butcher et al., 2002). The smaller quantity fed in this study may or may not have contributed to the negative result. By BLAST, ITS-1 sequence obtained from cat sarcocyst was not similar to any given for *Sarcocystis* spp. that utilize the opossum as a host. The opossum is therefore not likely to be a definitive host for *S. felis*. Although only one opossum was

used, such feeding trials are thought to be a sensitive bioassay for *S. neurona*, and comparable studies previously have been carried out with single opossums (Dubey et al., 2001b, Turay et al., 2002).

Biological diversity exists within *Sarcocystis* species; completion of the life cycle of *S. neurona* through cats, or other intermediate hosts, has not been successful with some horse CNS-derived isolates of *S. neurona*. In two studies, cat-derived *S. neurona* isolates were able to cause infection in cats or immune-deficient mice, but the horse-derived UCD-1 isolate of *S. neurona* was not infective (Butcher et al. 2002, Turay et al. 2002). This suggests cats may not be intermediate hosts for some isolates of *S. neurona* or the pathogenicity of the horse-derived isolates was insufficient. In addition, merozoites from a different horse-derived isolate of *S. neurona*, SN2, were not infective to raccoons (Dubey et al. 2002), whereas opossum-derived isolates were infective.

Two studies have shown cats do not function as definitive hosts for *Sarcocystis* spp. considered intermediate host species in cats. Immunosuppressed cats were dosed with sporocysts from an opossum naturally-infected with *S. neurona*. Muscle from these cats was then fed to two laboratory-raised domestic cats, neither of which shed sporocysts, demonstrating the cat is not a definitive host for *S. neurona* (Dubey et al., 2002). Sarcocysts found in Florida panthers and cougars were fed to two laboratory-reared domestic cats by Greiner and co-workers (1989). No sporocysts were shed from these cats, indicating the cat is not a definitive host for this species of *Sarcocystis*, later found to be *S. felis*.

The culture of bradyzoites was attempted in order to establish an isolate of *S. felis*. Such an isolate could then be used to develop additional molecular markers or foster

further biological study of this little-characterized *Sarcocystis* species. The cultured isolate, when fed to candidate host animals, might have been used to identify the definitive host for *S. felis*.

Mehlhorn and Heydorn (1979) and Fayer (1972) describe the introduction of *Sarcocystis* spp. bradyzoites into cell cultures, their subsequent differentiation into macro and microgamonts, sexual reproduction, and production of oocysts. These processes occurred quickly; oocysts were formed within one to three days in culture. This result was not achieved in the present study.

In this study, sera from 100 domestic cats were evaluated by immunoblot for *S. neurona* antibody; 5% were found to be positive. Sera from 196 domestic, pet cats in Michigan were evaluated for *S. neurona* antibody by both IFA and immunoblot (Rossano et al., 2002). Twenty-seven per cent of the samples were positive by IFA and 5% by immunoblot. Because pet cats were tested, seroprevalence may be lower than would be found in free-roaming cats, although many pet cats also have access to the outdoors. In the present study, 92% of cats were classified as "free-roaming", the remaining were surrendered pets. If serologic data is to be considered valid evidence of *S. neurona* infection, one might expect a higher prevalence of *S. neurona* antibodies in wild felid species or feral domestic cats due to greater exposure to their (unknown) definitive host(s). No serologic surveys for *S. neurona* antibodies have been conducted in wild felid species.

In an abstract presented at the 2001 Conference of Research Workers in Animal Diseases, Stanek and colleagues describe the use of the *S. neurona* agglutination test (SAT) to examine 36 cats living on horse farms where EPM had been diagnosed and 76

cats described as free-roaming. *Sarcocystis neurona* antibody was found in 39% of farm cats, and 16% of free-roaming cats (23% total). It is not known if any of the sera collected for the present study came from cats living on horse farms. Whereas the immunoblot test used by Rossano and colleagues (2002) utilized a slightly different test format and interpretation than the one used for the present study, significantly different test formats, reagents, and interpretation are used for the SAT and IFA tests, both of which reported higher seroprevalance. It is likely that differences in test formats and interpretation affect the number of samples that test positive or negative in each case.

To increase the integrity of the serological data, a commercial lab that performs immunobloting on mass scale was used to test cat sera from this study. The same laboratory performed immunoblotting of armadillo serum, assisting in the identification of the armadillo as a natural host for *S. neurona* (Tanhauser et al., 2001). However, the immunoblot test is not validated for use in samples from animals other than horses, and its utility for such samples is unknown. Though in the present study, immunoblotting was able to clearly demonstrate *S. neurona* antibody in the cat immunized with *S. neurona* merozoites. Histologic examination of postmortem CNS tissue is the "gold standard" for EPM diagnosis (Daft et al., 2002). According to Lindsay and Dubey (2001), the immunoblot is considered the "gold standard" of immunological-based testing for *S. neurona* IgG, and thus EPM. It is agreed however, that the test is best suited for use with CSF as an aid to diagnosis of EPM in horses with neurological disease; the test is not recommended for screening clinically-normal horses, as false positive results occur (MacKay et al., 2000, Lindsay and Dubey, 2001) in up to 40% of CSF samples from normal horses (Daft et al., 2002). Antigenic variation within *S. neurona* has been

reported. In non-commercial, research applications, immunoblotting of cat sera has demonstrated antigenic variation between several isolates of *S. neurona*. In contrast to positive sera from immunized rabbits, and infected mice and horses, serum from a sarcocyst-infected cat reacted against the 14-kDa band, but not 28-kDa band of antigen prepared from the UCD-1 isolate of *S. neurona*. However, when antigen prepared from another *S. neurona* isolate, MU1, was used, both 14-kDa and 28-kDa proteins were recognized by the cat serum (Turay et al. 2002). Further, banding patterns seen on immunoblots were different for mice inoculated with opossum-cat derived *S. neurona* merozoites (Sn-Mucat 2) and mice inoculated with the horse-derived UCD1 isolate (Butcher et al. 2002). Specific recommendations for the use of, and limitations associated with, immunobloting for *S. neurona* antibody may need to be considered when interpreting the serological data presented from this study and others evaluating cat sera.

In accordance with protocols for interpretation of equine sera (Neogen, Lexington, KY), cats from our study were considered positive if they reacted against a specific 17-kDa antigen of *S. neurona*. Likewise, sera were considered "non-specific" if they reacted only against a 30-kDa antigen. Inclusion of sera with both nonspecific or specific antibody reactivity would have yielded 19% cats positive for *S. neurona* antibody, a figure comparable with the 23% obtained from SAT as reported by Stanek and colleagues (2001). Greater seroprevalence reported by IFA may be due to cross-reactivity (Rossano et al, 2002).

This study found no apparent correlation between positive immunoblot result and presence of sarcocysts. These results could reflect problems with the sensitivity or specificity of the immunoblot in cats, or, more likely, visible sarcocysts were not

S. neurona. If the immunoblot was detecting *S. neurona* infections, *S. neurona* sarcocysts may not have been seen either because infections were immature (i.e., presarcocyst,), eliminated by the cats before encystment, or were too rare to be found by histologic techniques. It has been reported that *S. neurona* sarcocyst formation in the cat may take several months (Dubey et al., 2000). Identification of sarcocyst-infected muscle for further analysis may have been improved by muscle digestion, and examination of the digest for bradyzoites (Collins et al., 1980). Alternatively, lysis of muscle tissue and use of molecular markers specific for *S. neurona*, i.e. JNB 63/64 PCR primers followed by restriction enzyme digest (Tanhauser et al., 1999), may have been appropriate means to search for *S. neurona* infection in these cats.

Ostensibly, immunoblot results from this study might seem to indicate there is cross-reactivity of *S. felis* antibody with *S. neurona*. However, only one of five *S. felis* sarcocyst-positive cats was positive for *S. neurona* antibody by immunoblot; the other four cats were antibody-negative, suggesting cross-reactivity is not a factor. The serological data presented here have not resolved whether or not cats are natural hosts for *S. neurona*. However, as pointed out by Rossano and colleagues (2002), one might expect seroprevalence of *S. neurona* in intermediate hosts to be similar to that found in horses. This study found 5% of cats from this area of Florida to have *S. neurona* antibody. The seroprevalence in horses from this area is known to be much higher than 5%, perhaps as high as 50% (MacKay et al., 2000).

CHAPTER 6
SUMMARY AND CONCLUSIONS

Sarcocystis infection was present in the population of domestic cats sampled for this study. While the results presented here do not confirm a role for cats as intermediate host for *S. neurona*, the serologic data suggest that infection with *S. neurona* may occur; 5% of cats were seropositive. In contrast, sarcocysts commonly infected the cats; 10% were infected. Morphologic examination showed these sarcocysts to be *Sarcocystis felis*. Molecular analyses showed *S. felis* to be quite different from other *Sarcocystis* species for which comparable sequence data are available. Relatively little is known about the biology of *S. felis*. Further research is needed to identify the source of these infections and their pathogenicity in felids.

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BIOGRAPHICAL SKETCH

Karen Dunne Gillis was born October 20th, 1961 in Great Barrington, Massachusetts, the only child of Thomas and Kathleen Gillis. Karen grew up on a small farm in the village of Monterey, located in the Berkshire Hills of western Massachusetts. There, she developed a fondness for nature and animals that still continues. She attended public schools until grade 10, and then enrolled at the Berkshire School in Sheffield, Massachusetts. Her parents moved to south Florida, and Karen joined them there after graduation from Berkshire in 1979. Karen attended the University of Florida in Gainesville, graduating in 1984 with a Bachelor of Science degree from the College of Agriculture, majoring in both animal science and dairy science. Karen left the University environment for a short time, working in dairy management at farms in Georgia and Florida, but returned to Gainesville and the University of Florida in 1985 to work at the College of Veterinary Medicine. Karen remains happily and successfully employed there; she is currently a Senior Biological Scientist in the Department of Large Animal Clinical Sciences. Karen pursued her graduate studies while working full-time. After receipt of her master's degree in veterinary medical sciences, Karen plans to continue her work at the College of Veterinary Medicine.