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Review

A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM)

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Abstract

Equine protozoal myeloencephalitis (EPM) is a serious neurological disease of horses in the Americas. The protozoan most commonly associated with EPM is *Sarcocystis neurona*. The complete life cycle of *S. neurona* is unknown, including its natural intermediate host that harbors its sarcocyst. Opossums (*Didelphis virginiana*, *Didelphis albiventris*) are its definitive hosts. Horses are considered its aberrant hosts because only schizonts and merozoites (no sarcocysts) are found in horses. EPM-like disease occurs in a variety of mammals including cats, mink, raccoons, skunks, Pacific harbor seals, ponies, and Southern sea otters. Cats can act as an experimental intermediate host harboring the sarcocyst stage after ingesting sporocysts. This paper reviews information on the history, structure, life cycle, biology, pathogenesis, induction of disease in animals, clinical signs,

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1. Introduction and history

A clinical syndrome referred to as "segmental myelitis" was first described in detail by Rooney et al. (1970) based on 52 cases, 38 were horses from Lexington, KY, 12 were cases presented to the College of Veterinary Medicine at Philadelphia, PA, and two came from other sources. Protozoa were first reported in lesions from horses with these segmental lesions by three separate groups of researchers in 1974 (Cusick et al., 1974; Beech and Dodd, 1974; Dubey et al., 1974). The paper by Cusick et al. (1974) appeared first and it described clinical signs and gross lesions, and provided illustrations of the protozoan that are typical of what is now called equine protozoal myeloencephalitis (EPM). They misidentified the parasite as *Toxoplasma gondii*. It is clear now that the parasite reported by Cusick et al. (1974) was *Sarcocystis neurona* because they clearly illustrate a merozoite in Fig. 3 that does not contain rhoptries and Fig. 4 is a schizont undergoing division by endopolygeny. These structural features make the parasite a *Sarcocystis* species (see below). Beech and Dodd (1974) reported lesions and protozoa in eight horses, some of which were in the original report of Rooney et al. (1970). Dubey et al. (1974) reported lesions and protozoa in horses from Ohio and distinguished the parasite from *T. gondii*. Dubey (1974, 1976) re-examined the cases by Cusick et al. (1974) and Beech and Dodd (1974) and concluded that the parasite was not *T. gondii* and probably a *Sarcocystis* species. Simpson and Mayhew (1980) provided ultrastructural evidence that the protozoan causing EPM was a *Sarcocystis* species.

Beech (1974) called the condition "equine protozoan encephalomyelitis", but Mayhew et al. (1976) called it "equine protozoal myeloencephalitis", and this name has gained favor over the ensuing 25 years. These authors (Beech, 1974; Mayhew et al., 1976) also introduced the use of anti-protozoal therapy for the treatment of EPM (Table 1).

Attempts were made in the 1980s to reproduce EPM in horses experimentally by oral inoculation of oocysts or sporocysts of several different apicomplexan parasites, including *Sarcocystis* (Fayer and Dubey, 1986). These studies failed to determine the causative agent.

Cases of EPM were examined by one of us (JPD) from several different locations in North America. Structural studies of the parasites indicated that a single parasitic agent was present (Dubey et al., 1991a). The name *S. neurona* was proposed for the agent causing EPM in horses in 1991, and the parasite was isolated for the first time from a horse (Dubey et al., 1991a). The horse was from Ithaca, NY, and it had been given corticosteroids to increase the chance of isolating the parasite. Later that year, Davis et al. (1991a,b) isolated *S. neurona* from two horses from California and described its development in bovine monocyte cell cultures.

Antemortem diagnosis of EPM became feasible when Granstrom et al. (1993) developed a Western blot (immunoblot) test specific for *S. neurona* to detect antibodies in the serum

Table 1
History of *S. neurona* and EPM

Contribution	References
Clinical syndrome first recognized without a defined etiology	Rooney et al. (1970)
Protozoa first identified in lesions	Cusick et al. (1974), Beech and Dodd (1974), Dubey et al. (1974)
EPM defined as a disease	Beech (1974), Mayhew et al. (1976)
Anti-protozoal chemotherapy introduced	Beech (1974), Mayhew et al. (1976)
Protozoa causing EPM considered to be a *Sarcocystis* species	Dubey (1976), Simpson and Mayhew (1980)
Protozoa first isolated in cell cultures and named *S. neurona*	Dubey et al. (1991a)
In vitro development described	Davis et al. (1991a,b)
S. neurona Western blot developed for diagnosis	Granstrom et al. (1993)
PCR primers introduced for diagnosis and molecular characterizations	Fenger et al. (1994), Granstrom et al. (1994)
Opossum (*D. virginiana*) proposed as the definitive host for *S. neurona*	Fenger et al. (1995)
Clinical syndrome simulating EPM induced in horses by feeding sporocysts from opossums	Fenger et al. (1997a)
S. neurona separated from *S. falcatula* based on susceptibility of immunodeficient mice to *S. neurona*	Marsh et al. (1997a,b), Dubey and Lindsay (1998)
Opossum proven to be definitive host by inducing EPM-like disease in immunodeficient mice fed sporocysts	Dubey and Lindsay (1998)
In vitro and in vivo testing of chemotherapeutic agents began	Lindsay and Dubey (1999), Dubey et al. (2001b)
S. neurona isolated from CNS of a non-equine host	Lindsay et al. (2000b)
South American opossum (*D. albiventris*) from Brazil found to be another definitive host for *S. neurona*	Dubey et al. (2001c)
S. neurona sarcocyst discovered and life cycle completed	Dubey et al. (2000d)

and cerebrospinal fluid (CSF) of horses. Cell culture-grown merozoites are used as antigen, and the blots are probed with test serum or CSF or both. Antibody reactivity to *S. fayeri* or other important apicomplexan parasites of horses is differentiated. Presently two companies (EBI and Neogen) in Lexington, KY, and the Michigan State University Diagnostic Laboratory, utilize immunoblot testing commercially on horse serum and CSF. Recent serological surveys using the immunoblot method indicate that 30–50% of horses in US, Argentina, and Brazil have antibodies to *S. neurona* (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Dubey et al., 1999a,c; Tillotson et al., 1999).

The transmission and life cycle of *S. neurona* remained undetermined until 1995 when Fenger et al. (1995) proposed that the North American opossum (*Didelphis virginiana*) is a definitive host for the parasite based on ssuRNA gene sequence comparisons of cell culture-derived merozoites and sporocysts obtained from the intestines of opossums. Fenger et al. (1997a) induced clinical EPM in *S. neurona*-negative horses fed sporocysts collected from opossums. Horses fed sporocysts had antibodies to *S. neurona*, developed neurologic disorders and had lesions consistent with those seen in naturally affected horses. However, *S. neurona* was not demonstrated histologically or by cell culture assay (Fenger et al., 1997a). Dubey and Lindsay (1998) provided conclusive evidence that the opossum was the definitive host for *S. neurona*. They showed that interferon-gamma gene knockout (KO)

mice fed sporocysts from opossums developed neurological disorders similar to those seen in horses with EPM, demonstrated *S. neurona* in the tissues of the mice using immunohistochemistry, and recovered the parasites in cell culture. Merozoites collected from cell culture also produced encephalitis in KO mice, thus fulfilling Koch's postulates (Dubey and Lindsay, 1998). The life cycle of *S. neurona* was completed when sarcocysts were found in muscles of domestic cats (*Felis domesticus*) fed sporocysts from opossum feces; laboratory-raised opossums fed infected cat muscles shed *S. neurona* sporocysts (Dubey et al., 2000d).

Confusion arose in 1995 regarding the identity of *S. neurona* and the role of birds in the life cycle of *S. neurona* because of close molecular similarities in the ssuRNA genes of *S. neurona* and *Sarcocystis falcatula*. *S. falcatula* has birds as intermediate hosts and the opossum (*D. virginiana*) as the definitive host. Dame et al. (1995) concluded that *S. neurona* should be a synonym of *S. falcatula* after comparing the ssuRNA sequence of *S. falcatula* bradyzoites with the ssuRNA genes sequence of *S. neurona* merozoites. In retrospect, the inocula used by Fenger et al. (1997a) may have contained both *S. neurona* and *S. falcatula*. Marsh et al. (1997a), Dubey and Lindsay (1998), and Lindsay et al. (1999) provided evidence that *S. neurona* and *S. falcatula* were structurally and biologically different. Cutler et al. (1999) provided additional evidence that *S. neurona* and *S. falcatula* were not the same parasite. They determined that seroconversion to *S. neurona* antigens does not occur after horses are fed sporocysts of *S. falcatula* collected from the intestines of opossums (Cutler et al., 1999). *S. neurona* was recently isolated from sporocysts from the South American opossum, *Didelphis albiventris*, from Brazil (Dubey et al., 2001c). Additional historical features are summarized in Table 1.

2. Host range, parasite structure, life cycle, and molecular biology

2.1. Host range

S. neurona is a coccidian (Apicomplexa: Sarcocystidae) with opossums as the definitive hosts (Fenger et al., 1997a; Dubey and Lindsay, 1998; Dubey et al., 2000d, 2001a) and a variety of mammals as aberrant intermediate hosts (Fig. 1). The natural intermediate host is not known. *S. neurona*-like infections have been reported in raccoons (Dubey et al., 1990, 1991b; Stoffregen and Dubey, 1991; Thulin et al., 1992; Hamir and Dubey, 2001), a domestic cat (Dubey et al., 1994; Dubey and Hamir, 2000), two mink (Dubey and Hedstrom, 1993; Dubey and Hamir, 2000), skunks (Dubey et al., 1996; Dubey and Hamir, 2000), a pony (Dubey and Miller, 1986; Dubey and Hamir, 2000), a zebra (Marsh et al., 2000), Pacific harbor seals (Lapointe et al., 1998), and Southern sea otters (Rosonke et al., 1999; Lindsay et al., 2000b); infections were diagnosed using immunohistochemical methods for *S. neurona*. *S. neurona*-like infection was also reported in a monkey by Klumpp et al. (1994) but the identification was not confirmed by *S. neurona* immunohistochemistry (Dubey and Hamir, 2000). *S. neurona* has only been isolated from the CNS of horses (Dubey et al., 1991a) and from a Southern sea otter (Lindsay et al., 2000b). *S. neurona* strains from horses, opossums, and the sea otter are molecularly identical (Tanhauser et al., 1999; Dubey et al., 2001a,c,d; Rosenthal et al., 2001).

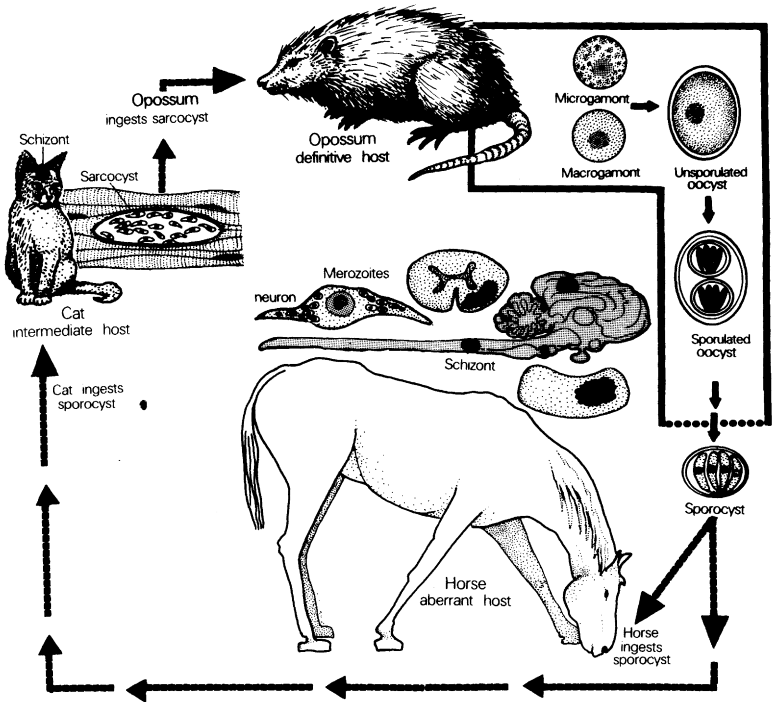

Fig. 1. Life cycle of *Sarcocystis neurona*. Opossums are the definitive hosts and horses are aberrant hosts; natural intermediate hosts are unknown. *S. neurona* parasitizes and causes lesions (dark areas) in the brain and spinal cord of horses. Only schizonts and merozoites are found in the aberrant hosts. Cats can act as an experimental intermediate host. Both schizonts and sarcocysts are found in tissues of cats. Opossums excrete sporulated oocysts or sporocysts after ingesting muscles infected with sarcocysts.

Clinical EPM infections in horses (Fig. 2) have been reported from US and Canada (Clark et al., 1981; Fayer et al., 1990), Brazil (Barros de Lambardo et al., 1986; Masri et al., 1992) and Panama (Granstrom et al., 1992). We are not aware of confirmed diagnosis of EPM from Mexico.

2.2. Parasite and life cycle

Only asexual stages have been identified in the aberrant intermediate hosts, and they are confined to the brain and spinal cord, and any part of the central nervous system (CNS) may be affected. The number of asexual generations (schizogony) has not been determined. Apparently, *S. neurona* can multiply in the CNS of equids for many months. Both neural and inflammatory cells in the CNS may be parasitized (Fig. 3). As many as 13 schizonts were found in a single neuron and several hundred merozoites (Fig. 3) may be present in one neuron (Dubey et al., 1974). In histologic sections of CNS, individual merozoites are about 3–5 μm long and contain a single, centrally located vesicular nucleus (Fig. 3). *S. neurona* multiplies in the CNS by a specialized form of schizogony called endopolygeny (Fig. 4).

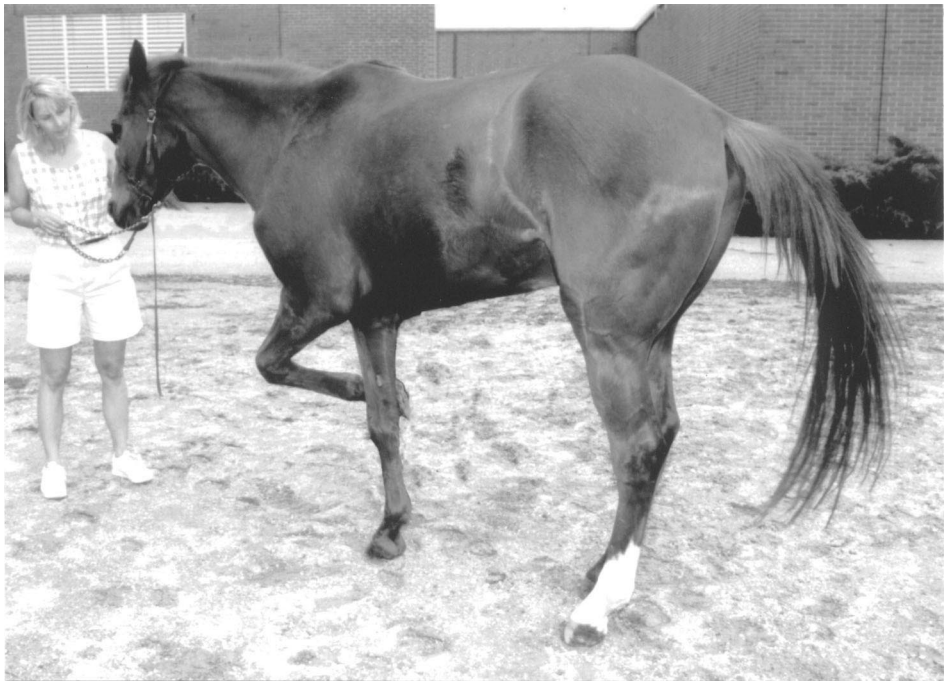

Fig. 2. An 18 year-old Quarter Horse gelding with ataxia and atrophy of hind quarter muscles. This horse improved after anti-protozoal therapy for EPM and then relapsed 1 year later to the present state.

In endopolygeny, the nucleus becomes lobulated. The lobes are connected by chromatin strands, and the lobes may be arranged in groups (Fig. 4B). During the development of merozoites to schizonts, the merozoite nucleus enlarges, and the nucleus divides into four or more nucleoli (Fig. 4A). In early stages the uninucleate schizont sometimes resembles a macrophage or a degenerated host cell. Finding multiple nucleoli in a nucleus helps to distinguish *S. neurona* from degenerating host cells. Apparently several merozoites may develop within the same host cell (Dubey et al., 1991a) to become mature schizonts and produce merozoites without leaving the host cell (Fig. 4C). Thus, the developmental cycle may be asynchronous. Merozoites are formed centrally or peripherally in the schizont, often around a residual body. Schizonts and merozoites are periodic acid Schiff (PAS) reaction negative. Mature schizonts in the CNS are up to 30 μm long and they may be oval, round, elongated or irregular in shape.

The natural intermediate hosts of *S. neurona* are unknown. The sarcocyst stage of *S. neurona* and experimental intermediate were recently identified (Dubey et al., 2000d). Laboratory-raised cats fed sporocysts from a naturally infected opossum developed sarcocysts in their skeletal muscles. The sarcocysts were microscopic (∼700 μm long) with a 1–2 μm thick cyst wall. The bradyzoites were slender and tiny (∼5 μm long). Laboratory-raised opossums fed infected cat muscles shed sporocysts. The sporocysts were $\sim 10 \times 8$ μm.

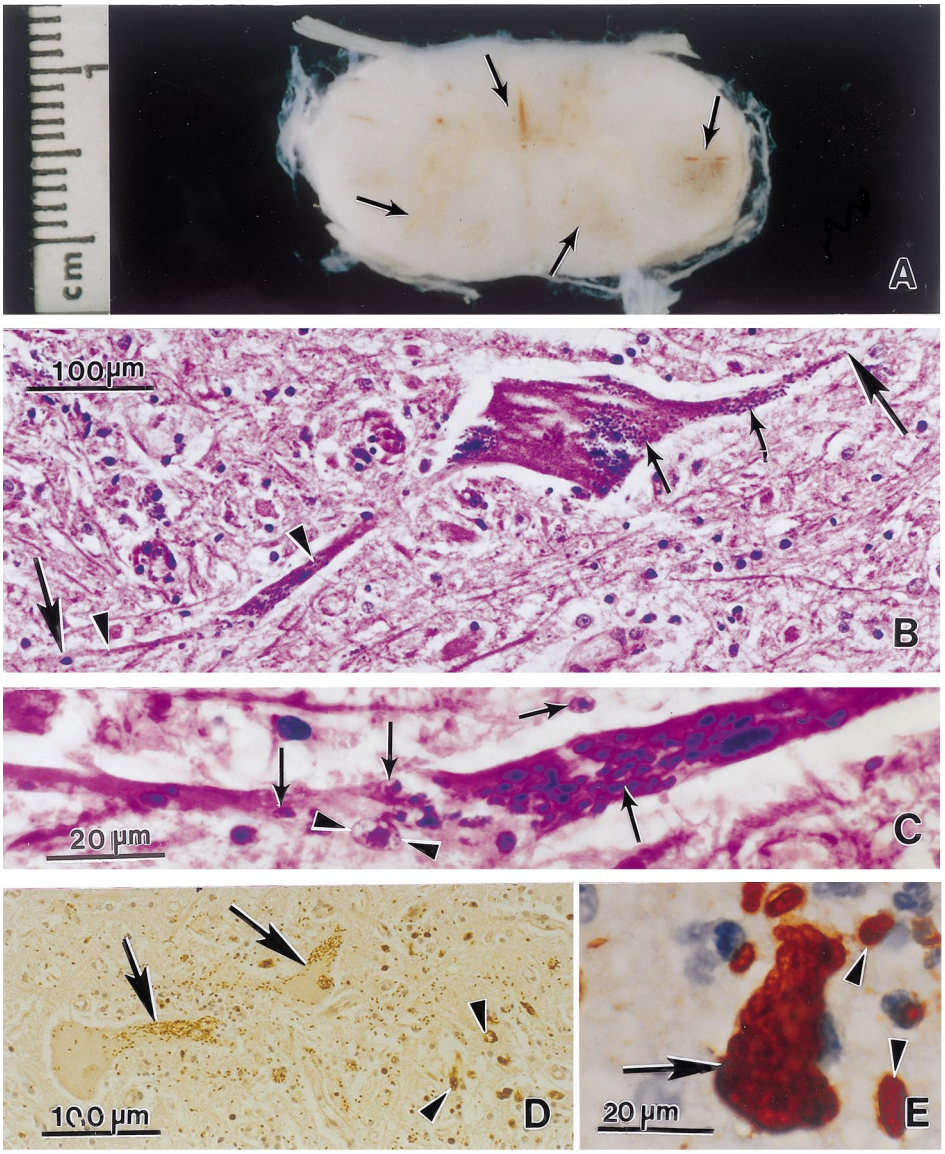

Fig. 3. Lesions and *S. neurona* in sections of spinal cord of horses. (A) Focal areas of discoloration (arrows). Unstained. *S. neurona* (SN2 isolate) was obtained from this horse (Davis et al., 1991a). (B) A heavily parasitized neuron (large arrows). Area between arrowheads is enlarged in Fig. 3C. H&E. (C) Intraneuronal and extracellular merozoites (arrows). Note one young schizont (arrowheads) with an undivided nucleus. H&E. (D) Heavily infected neurons (arrows). Immunohistochemical stain with anti-*S. cruzi* serum. All brown spots (arrowheads) are organisms. (E) Schizonts (arrow) and merozoites (arrowheads). Immunohistochemical stain with anti-*S. neurona* serum. (B)–(E) are from the horse from which *S. neurona* was named (Dubey et al., 1991a).

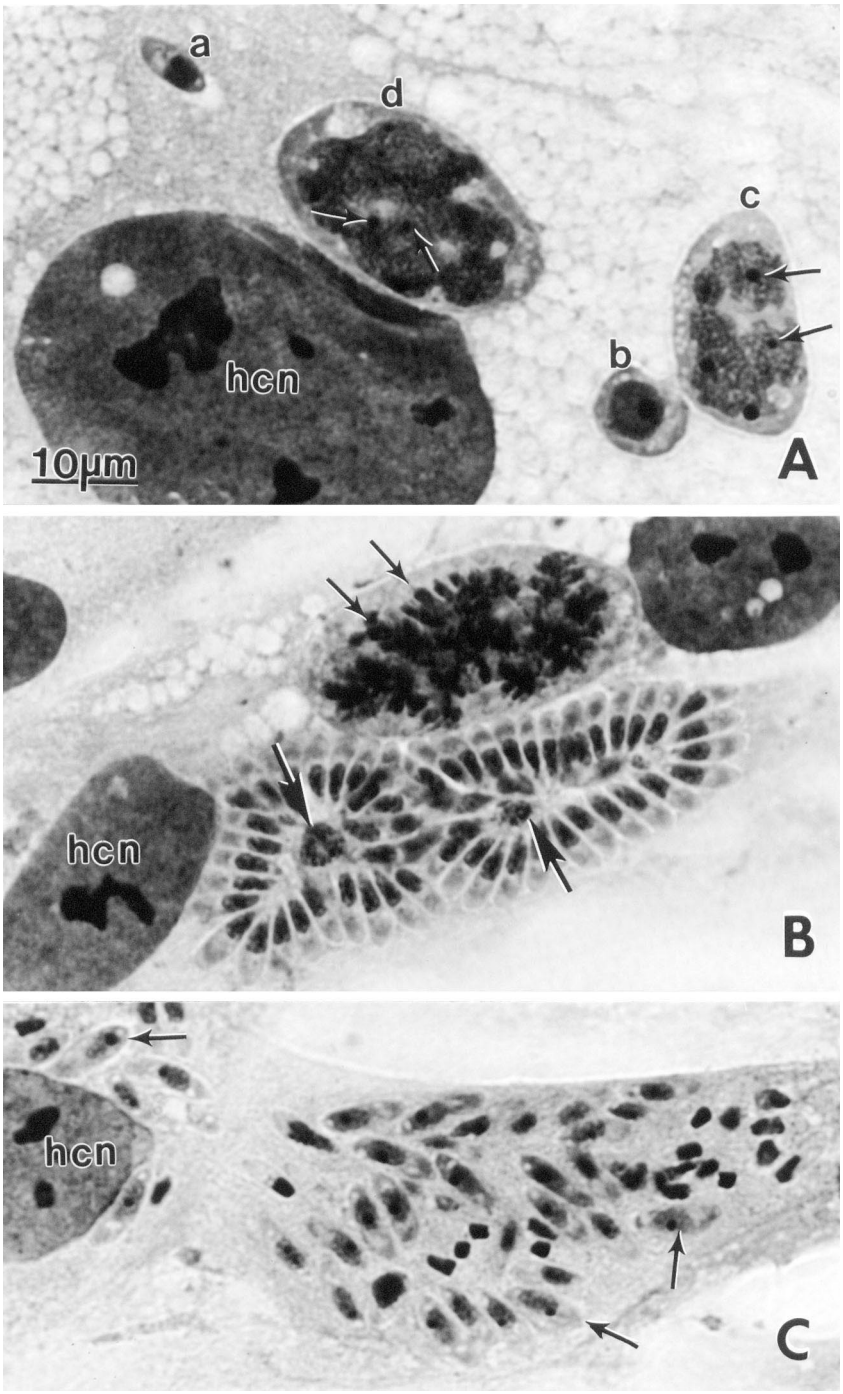

Fig. 4.

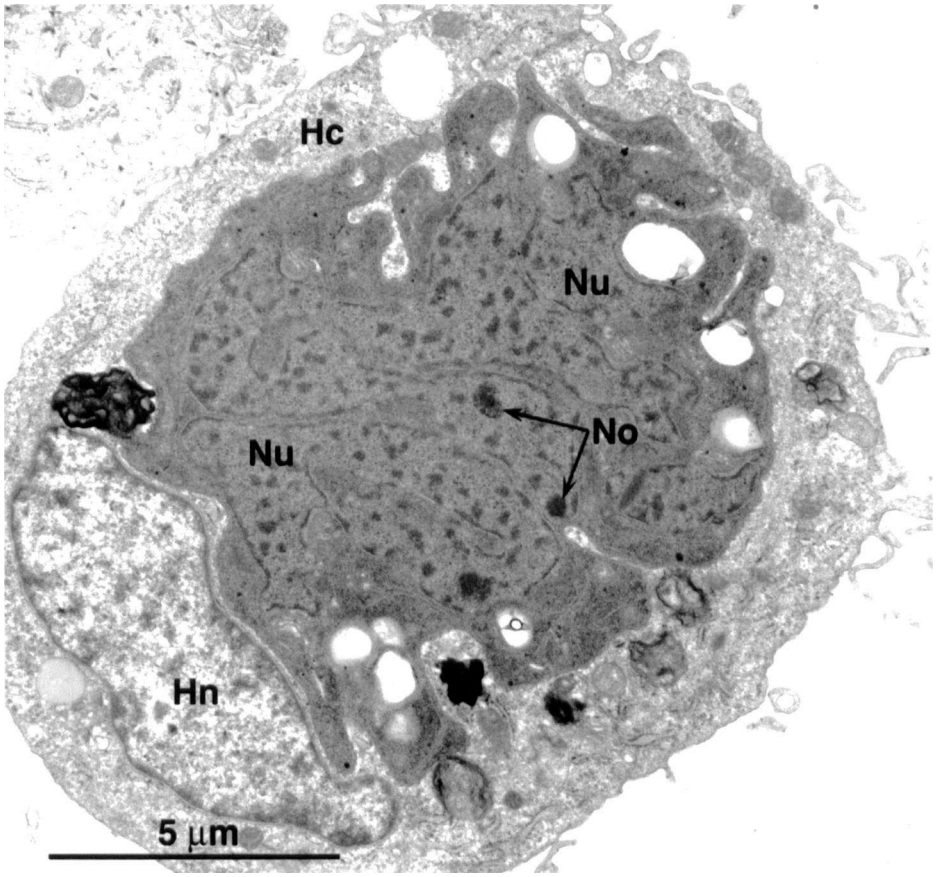

Fig. 5. Transmission electron micrograph of an immature schizont of *S. neurona* in a cultured bovine monocyte. The schizont is free in the host cell cytoplasm (Hc) without a parasitophorous vacuole and has a lobulated nucleus (Nu) containing several nucleoli (No). Hn: host cell nucleus.

Sporocysts from experimentally infected opossums were infective to gamma interferon KO mice and ponies (Dubey et al., 2000d).

In addition to the North American opossum (*D. virginiana*), the South American opossum (*D. albiventris*) has recently been found to be another definitive host for *S. neurona*, *S. falcatula*, *S. speeri* and other, unnamed species (Dubey et al., 2000b,c,e, 2001a,c).

Fig. 4. Schizogonic stages of *S. neurona* (SN2 isolate) in bovine turbinate cells. Giemsa stain. Bar applies to all figures. (A) Differentiation of merozoite nucleus into schizont nucleus. Note a merozoite with enlarged nucleus (a), globular schizont with nucleus containing one nucleolus (b), lobulated nucleus (c) with five nucleoli (arrows), and a schizont with large nucleus with two nucleoli (arrows) (d). (B) Three schizonts. Note merozoites budding at the surface (small arrows) and residual bodies (large arrows) in two mature schizonts. (C) Development of second generation of schizonts without merozoites of the first generation leaving the host cell. Arrows point to merozoites transforming to schizonts. Hcn: host cell nucleus.

2.3. Ultrastructure

Ultrastructurally, schizonts and merozoites are located in the host cell cytoplasm without a parasitophorous vacuole at any stage of development (Fig. 5). Merozoites develop by endopolygeny, a form of schizogony in which numerous merozoites initiate development internally and later bud at the surface of the schizont (Figs. 5 and 6). After the schizonts reach a particular size, merozoites appear to develop essentially synchronously with two merozoites developing above each lobe of the nucleus (Speer and Dubey, 2001).

Some merozoites escape from their host cells, penetrate other cells, and undergo additional schizogonous generations. However, most merozoites remain within the host cell in which they had originally developed and initiate another generation of schizogony (Fig. 7). Only a few of these actually develop to mature schizonts.

Fully formed merozoites have a pellicle, numerous polysomes and ribosomes, smooth and rough endoplasmic reticulum, 22 subpellicular microtubules, 9–16 dense granules, 25–75 micronemes, a plastid, a Golgi complex, 1–3 mitochondria, a conoid, 2 apical rings, 2 polar rings, 0–6 lipid bodies, and a nucleus and nucleolus (Speer and Dubey, 2001). Rhoptries are absent (Fig. 6B). Most micronemes are located anterior to the nucleus including 1–6 micronemes in the conoid. Merozoites are either slender ($7.3 \times 1.7\ \mu m^2$) or stumpy ($7.7 \times 3.1\ \mu m$). Dense granules appear to arise from the maturation face of the Golgi complex. The ultrastructure of in vitro derived schizonts and merozoites are similar to in vivo derived organisms (Speer and Dubey, 2001). *S. neurona* sarcocysts from experimentally infected cat muscles were microscopic and had slender villar protrusions (Vp) on cyst walls (Fig. 8). The Vp were up to 2.5 μm long and had microtubules that extended into the ground substance (Fig. 8).

2.4. Molecular biology

The first genetic analysis of *S. neurona* was done using a random amplified polymorphic DNA assay to compare *S. neurona* to *Sarcocystis* spp., *T. gondii*, and several *Eimeria* spp. (Granstrom et al., 1994). A random primer was identified that clearly differentiated *S. neurona* from the other species tested. Subsequently, the nuclear small subunit-ribosomal (nss-r) RNA gene was amplified from cultured *S. neurona* merozoites, cloned, and sequenced (Fenger et al., 1994). Sequence information was used to design a polymerase chain reaction (PCR) assay to screen DNA extracted from sporocysts shed by various candidate definitive hosts. Sporocysts collected from opossums (*D. virginiana*) were identified using the PCR assay (Fenger et al., 1995). The nss-rRNA gene from sporocyst DNA was cloned and sequenced and found to be virtually identical to the nss-rRNA gene sequence of *S. neurona*. Following identification of the opossum as the definitive host, the nss-rRNA gene sequence of *S. falcatula*, which cycles between opossums and various birds, was determined (Dame et al., 1995). Although the sequences were virtually identical, subsequent biologic, morphologic, and molecular evidence showed the two species were distinct (Marsh et al., 1997a; Dubey and Lindsay, 1998; Cutler et al., 1999; Lindsay et al., 1999; Tanhauser et al., 1999).

Analyses of various genomic DNA sequences showed that opossums are host to both *S. neurona* and *S. falcatula* (Marsh et al., 1999a; Tanhauser et al., 1999). A PCR test,

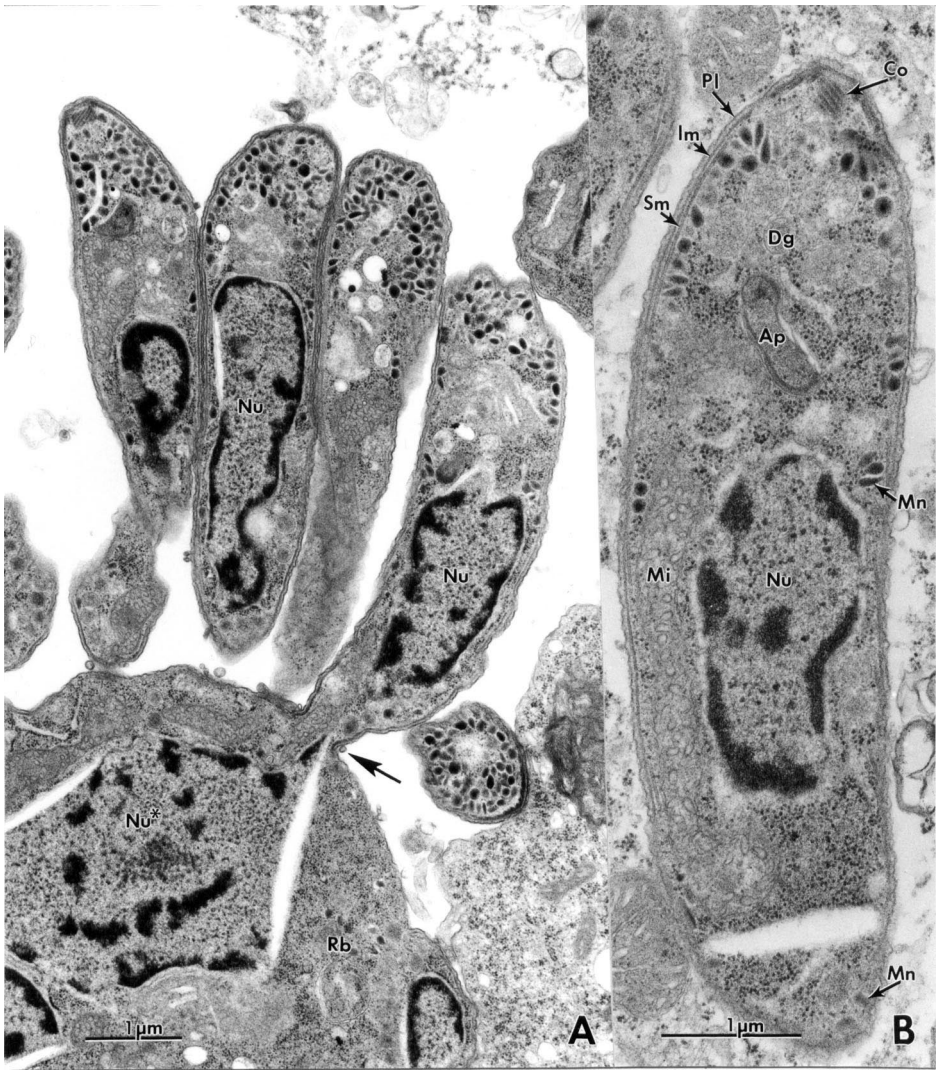

Fig. 6. Transmission electron micrographs of *S. neurona* in the brain of a horse. *S. neurona* (isolate SN7) was obtained from this horse (Dubey et al., 2001d). (A) Portion of a schizont showing several budding merozoites, one of which is still attached (arrow) to the residual body (Rb); Nu, nucleus of merozoite; Nu*, nucleus of schizont still in residual body. (B) Merozoite, Ap, putative apicoplast (=Golgi adjunct); Co, conoid; Dg, dense granule; Im, inner membrane complex; Mi, mitochondrion; Mn, microneme; Pl, plasmalemma; Sm, subpellicular microtubule.

designed using appropriate sequence differences, was used to screen opossums in Florida and two additional unknown *Sarcocystis* spp. were identified (Tanhauser et al., 1999).

Phylogenetic analyses of the nss-rRNA genes from multiple members of the family Sarcocystidae have been analyzed to study relatedness and to help complete parasite life cycles. Initially, it was believed that protozoa co-evolved with the definitive host (Barta, 1989).

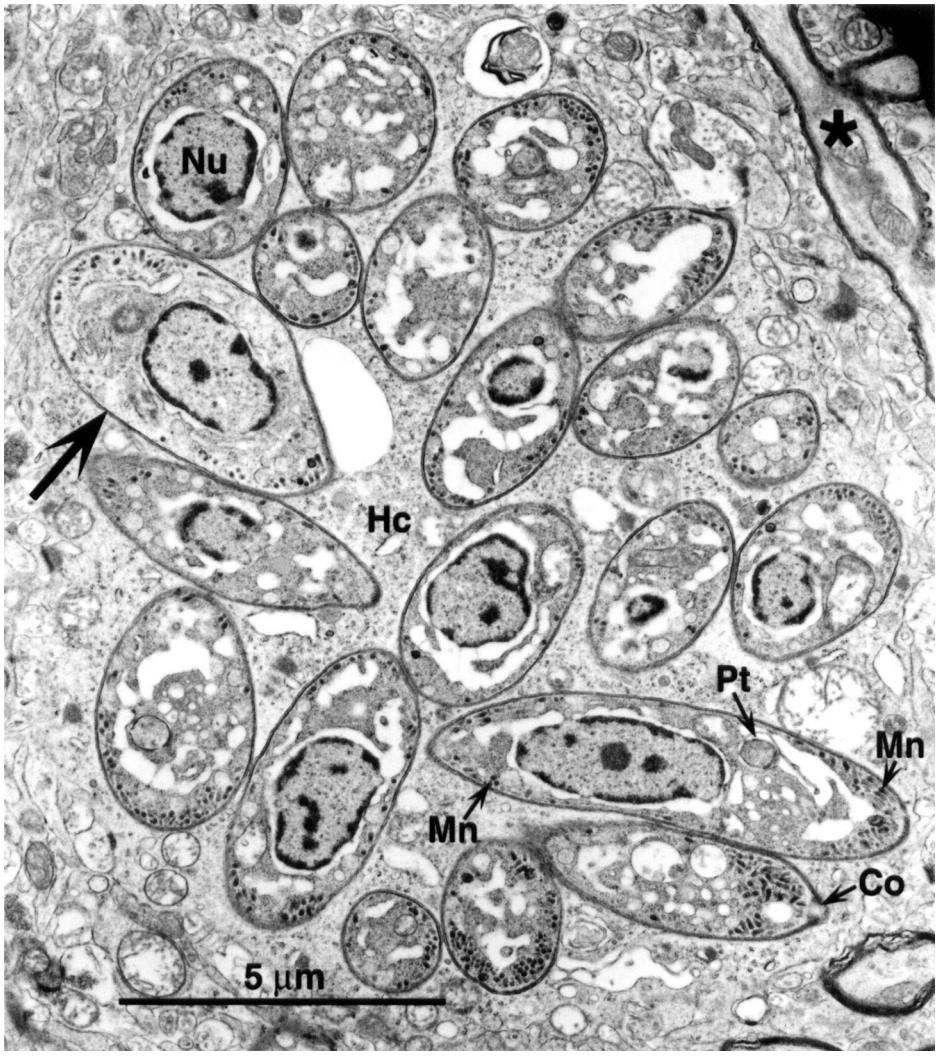

Fig. 7. Transmission electron micrograph of a mature schizont of *S. neurona* in a neuron in the brain of a KO mouse, 50 days after subcutaneous injection with merozoites of SN6 isolate of *S. neurona*. The merozoites are scattered free in the cytoplasm (Hc) of the host cell and one (arrow) of the merozoites is in an early stage of schizogony. Merozoites show micronemes (Mn) at each pole. A myelinated cytoplasmic process (★) of a neuron is visible in the upper right corner. Co, conoid; Pt, plastid.

Phylogenetic models suggested that the Sarcocystidae could be divided into two major divisions or clades dependent upon co-evolution with either a felid or canid definitive host (Tenter et al., 1992). The nss-rRNA gene sequence of *S. neurona* placed it in the felid clade (Fenger et al., 1994). A more recent analysis using nss-rRNA gene sequences from many more *Sarcocystis* species identified two clades among *Sarcocystis* spp. based on ruminant

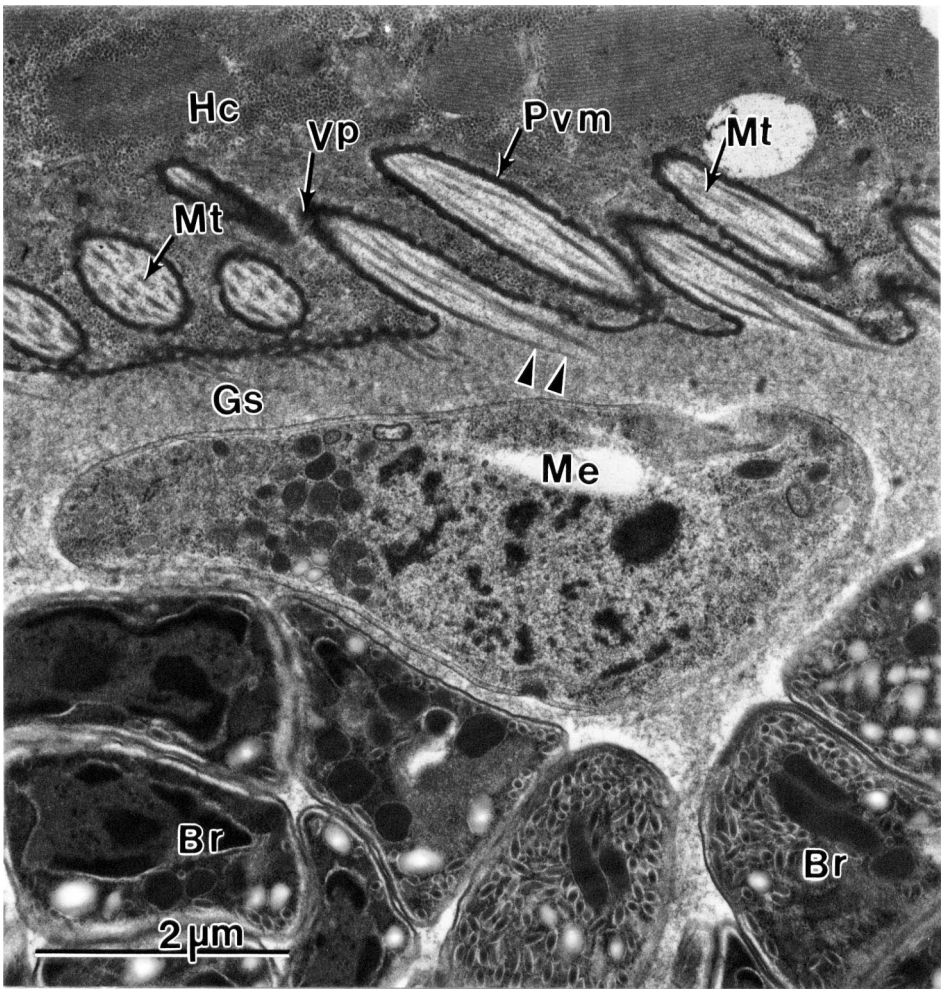

Fig. 8. Transmission electron micrograph of a sarcocyst in skeletal muscle of cat 525, 142 days after feeding sporocysts. Note wavy parasitophorous vacuolar membrane (Pvm), Vp projecting into host cell (Hc), microtubules (Mt) from tip to the base of Vp, and extending (arrowheads) into ground substance (Gs). Also note a metrocyte (Me) and several bradyzoites (Br).

or non-ruminant intermediate host utilization (Jenkins et al., 1999). Species using ruminant intermediate hosts separated into two monophyletic or single-origin clades containing organisms using either felid or canid definitive hosts (Holmdahl et al., 1999). *S. neurona* was placed in the non-ruminant clade and was most closely related to *S. mucosa*, a parasite believed to use the Bennetts wallaby (*Dayrus* sp.) as a definitive host (Jakes, 1998). The Bennetts wallaby, a carnivorous marsupial found in Australia, is closely related to the opossum.

Neospora sp. has been identified in four cases of EPM (Marsh et al., 1996a; Daft et al., 1997; Hamir et al., 1998; Cheadle et al., 1999). A new species, *Neospora hughesi*, was

isolated from an affected horse and distinguished from *Neospora caninum* based on nucleotide sequence differences in the first internal transcribed spacer (ITS-1) region of the rRNA gene (Marsh et al., 1998). Amino acid sequence differences also were identified in two immunodominant surface antigens (NcSAG1 and NcSRS2) of *N. hughesi* and *N. caninum* (Marsh et al., 1999b).

3. Pathogenesis

The pathogenesis of EPM is not clear because the complete life cycle is unknown. *S. neurona* can parasitize all regions of the CNS, from the anterior of cerebrum to the end of spinal cord. There is no report of *S. neurona* within peripheral nerves. *S. neurona* schizonts and merozoites are found in neurons, mononuclear cells, glial cells, and perhaps other neural cells (Dubey et al., 1974). The route of migration of the parasite from the time of ingestion of sporocysts to parasitism of the CNS is unknown. Studies in gamma interferon gene KO mice fed *S. neurona* sporocysts indicate that *S. neurona* initially multiplies to a limited extent in visceral tissues and then is transported to the CNS probably via leukocytes (Dubey, 2001). Three weeks after infection, *S. neurona* is mainly confined to the CNS (see Section 9.1).

Clinical signs of EPM are dependent on the area of the CNS parasitized. For example, involvement of the cerebrum may cause depression, behavior changes or seizures. Lesions in the brainstem and spinal cord may cause gait abnormalities, incoordination caused by involvement of ascending and descending tracts, and any of a variety of signs attributable to damaged cranial nerve nuclei (MacKay et al., 1992; Divers et al., 2000). These signs include facial nerve paralysis, head tilt, ataxia of one or more limbs (Fig. 2), and a tendency to lean to one side, paralysis of the tongue, urinary incontinence, dysphagia, and atrophy of masseter-temporal muscles (Beech, 1974; Mayhew et al., 1976; Traver et al., 1978; Clark et al., 1981; Barros de Lambardo et al., 1986; Madigan and Higgins, 1987; Brewer and Mayhew, 1988; MacKay et al., 1992; Ronen, 1992; Dunigan et al., 1995; Moore et al., 1995, 1997; Sedrish and Ramirez, 1996; MacKay, 1997; Fenger et al., 1997b; Scarratt and Wallace, 1998; Scarratt et al., 1999; McClure and Palma, 1999). Severe damage in the gray matter that innervates muscles of the limbs can produce weakness and atrophy of innervated muscles. The quadriceps and gluteal muscles are often atrophied (Fig. 2); *S. neurona* has not been found in muscles.

Factors governing severity of EPM are unknown. Clinical EPM is often reported in well cared for race horses 3–6 years of age. Clinical EPM does not seem to be associated with poor nutrition or known concurrent infections. There are no confirmed reports of clinical EPM in horses younger than 6 months of age. Although there is mention of a case in a review paper (Fayer et al., 1990), there was no histologic confirmation of CNS infection.

4. Clinical signs

EPM is often a progressively debilitating disease affecting the CNS of horses (Beech, 1974; Brewer and Mayhew, 1988; MacKay et al., 1992; Granstrom and Reed, 1994; MacKay, 1997). The clinical signs may vary from acute to insidious onset of focal or multifocal signs

of neurologic disease involving the brain, brainstem, spinal cord or any combination of the areas of the CNS. Some horses affected with EPM have abnormal upper airway function, unusual or atypical lameness or even seizures. In severe cases, the horse may have difficulty with standing, walking, or swallowing, and the disease may progress very rapidly. In some horses, the disease appears to stabilize or remain static for a time period.

The early clinical signs of stumbling and frequent interference are often easily confused with a lameness of either the thoracic and/or the pelvic limbs. In many horses the disease tends to have a gradual progression of clinical signs including ataxia, but in some horses there may be mild clinical signs followed by a rapidly progressive course. On physical examination, the vital signs are usually normal, although some horses may appear thin and mildly depressed. Neurological examination often reveals an asymmetric weakness, ataxia, and spasticity involving all four limbs. Frequently, areas of hypalgesia or complete sensory loss may be noted. The most frequent brain or cranial nerve deficits observed in horses appear to be head tilt, depression, facial nerve paralysis, and difficulty in swallowing, although signs are not limited to these areas. Gait abnormalities are often a result of damage to the spinal cord and may be quite variable depending on the location and severity of the lesion.

Most horses affected with EPM are bright and alert; however, any horse with signs of neurologic disease is a candidate to have EPM. At the time of initial examination, most horses have normal blood values. One of the most helpful clinical signs is that horses with EPM often have asymmetric gait deficits with focal muscle atrophy. This can be a useful differentiating feature and may help distinguish EPM from some of the other neurological diseases.

Cervical vertebral myelopathy (CVM) is one of the more common neurologic diseases affecting the spinal cord only (Hamir et al., 1992; Moore et al., 1995). This is caused by stenosis of the vertebral canal and may sometimes be accompanied by instability between the vertebrae. CVM occurs most commonly in horses 1–3 years of age. It is more common in males and usually presents with symmetric signs, with the pelvic limbs more affected than the thoracic limbs. Equine herpes virus myelitis (EHV-1) is a viral vasculitis of horses, which can affect any breed. There is usually a prior history of abortion and/or mild respiratory disease on the farm, which can affect a number of horses. It is usually symmetric with primary rear limb weakness and ataxia, urine dribbling and sometimes fecal retention. Occasionally horses with EHV-1 may be recumbent and are unable to rise. Muscle wasting, along with progressive weakness, may be an early sign of equine motor neuron disease (EMND) which can initially appear with signs similar to those demonstrated by horses with EPM.

5. Diagnosis

Although many neurologic disorders affect the horse, EPM remains the most commonly diagnosed infectious equine neurologic disease in America (Boy et al., 1990; Hamir et al., 1992). A complete neurologic examination and implementation of a thorough diagnostic plan to rule out differential diagnoses are essential prerequisites to laboratory testing and appropriate interpretation of clinical signs. Many ancillary diagnostic procedures may be required to differentiate primary muscloskeletal disorders and other neurologic diseases from EPM.

5.1. Blood chemistry

EPM does not produce consistent detectable changes in complete and differential cell counts or serum chemistry values (MacKay, 1997). Nonetheless, these assays may help to differentiate other diseases. In addition, EPM does not produce consistent changes in CSF color, clarity, cell counts, or the concentrations of antibody, protein, enzymes, glucose, and electrolytes. Analysis of CSF may be useful for differentiation of several infectious and non-infectious neurologic diseases (Freeman et al., 1989). Iatrogenic blood contamination of CSF during sample collection is common and precludes useful analysis. The spinal tap may be repeated in few days to avoid confounding results. Miller et al. (1999) nicely demonstrated impact of even minuscule amount of blood contamination of CSF on misdiagnosis of *S. neurona*.

5.2. Albumin quotient

The ratio of albumin concentration in CSF and serum (albumin quotient: AQ) may be useful to assess CSF sample quality (Andrews et al., 1995). Albumin is the most abundant serum protein, but it is not produced in CSF and must leak in from the general circulation. Total albumin concentration in CSF and the AQ can be compared to established normal ranges to help assess the integrity of the blood brain barrier. If the total albumin concentration in CSF and/or AQ are elevated, increased blood brain barrier permeability or accidental blood contamination of the sample are likely.

The ratio of total IgG concentration in CSF and serum can be used in conjunction with the AQ to evaluate intrathecal IgG production and to further evaluate blood brain barrier integrity (IgG index) (Andrews et al., 1995). However, index sensitivity of this test has been questioned (Cohen and McKay, 1997; Miller et al., 1999). Since immunoblot analysis (see below) for detection of anti-*S. neurona* antibodies in CSF is more sensitive than methods used to determine total albumin concentration, AQ, or IgG index, these indices may fail to detect blood contamination or a loss of blood–brain barrier integrity, which would confound immunoblot interpretation. Highly immunoreactive blood presents the greatest risk (Miller et al., 1999). Although the presence of elevated albumin in CSF and AQ remain useful, results within normal limits must be interpreted with caution.

5.3. Immunoblot analysis

The immunoblot test detects the presence of *S. neurona*-specific antibodies in serum and CSF (Granstrom et al., 1993). It was developed to differentiate antibodies produced against *S. neurona* and *S. fayeri* (a *Sarcocystis* species with a horse–dog cycle). Antibody cross-reactivity among *Sarcocystis* spp. in the indirect fluorescent antibody test or hemagglutination test has been recognized for many years (Mayhew et al., 1978). The immunoblot test was developed in 1991 using cultured *S. neurona* merozoites and antisera from horses with histologically confirmed EPM or experimental *S. fayeri* infection, and polyclonal rabbit antisera against *S. neurona*, *S. cruzi*, and *S. muris* in an immunoblot format. The reactivity of the various sera to the protein profile of *S. neurona* were compared. Eight proteins were

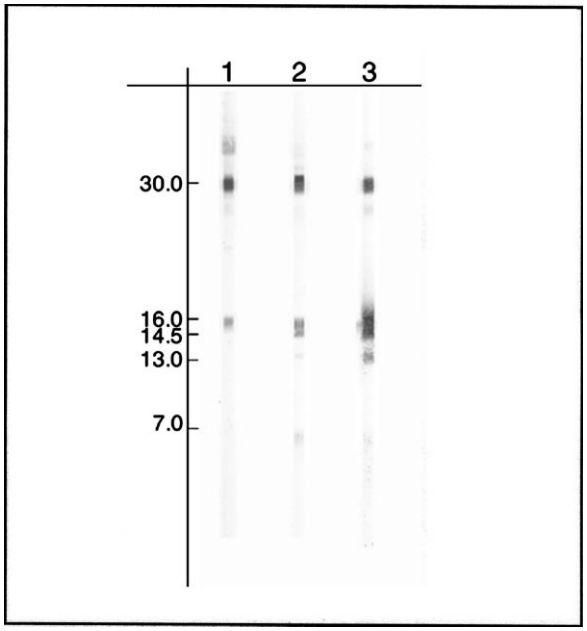

Fig. 9. Standard immunoblot test (no pre-incubation with bovine serum) using solubilized *S. neurona* merozoites separated in a 10–20% linear gradient SDS–PAGE gel. The blot was probed with serum from three horses. Lane 1 demonstrates a negative pattern of reactivity (30.0 and 16.0 kDa protein bands only); Lane 2 demonstrates a positive pattern of reactivity (14.5, 13.0, and 7.0 kDa bands); Lane 3 represents a positive pattern of reactivity (14.5 and 13 kDa protein bands).

recognized only by antibodies produced in horses with EPM or horses and rabbits injected with *S. neurona*. A subset of these proteins became the basis of interpretation for the standard EPM test. Specific and non-specific *S. neurona* proteins have been assigned varying molecular weights dependent on the conditions of electrophoretic separation and molecular weight markers used (Granstrom et al., 1993; Marsh et al., 1996b; Liang et al., 1998; Dubey et al., 1999a; Rossano et al., 2000). The current system used by Equine Biodiagnostics Incorporated (EBI, Lexington, KY) for commercial testing utilizes 10–20%, 15 cm polyacrylamide gradient gels to achieve optimal protein separation. Molecular weights are assigned using Bio-Rad broad range, pre-stained molecular weight markers. Under these conditions, the specific proteins used for diagnostic testing have relative mobilities of 14.5, 13, and 7 kDa (Fig. 9). The 13 kDa band actually represents a closely associated doublet of specific proteins. Immunodominant bands at 30 and 16 kDa are non-specific proteins. Additional non-specific bands frequently appear between 14.5 and 13 kDa bands and the 13 and 7 kDa bands which may confuse inexperienced workers.

A recent report suggests a modification that reportedly boosts test sensitivity and specificity to nearly 100% (Rossano et al., 2000). This assertion is based on the elimination of cross-reactivity with *S. cruzi* (cattle–dog cycle). In the modified test, immunoblots are incubated with sera from cattle naturally exposed to *S. cruzi* prior to the addition of equine

serum and CSF samples. The standard test, as noted above, was developed by excluding *S. neurona* proteins that cross-react with antisera against *S. cruzi*, *S. muris*, and *S. fayeri*.

The basis of the reported enhancement appears to be undefined nonspecific factors. *S. cruzi* does not infect the horse (Dubey et al., 1989). It is doubtful that horses produce anti-*Sarcocystis* antibodies following ingestion of sporocysts specific for other host species. For example, sporocysts from *S. falcatula*, a species more closely related to *S. neurona*, failed to elicit a detectable antibody response when fed to horses (Cutler et al., 1999). Although *S. cruzi* and *S. fayeri* share antigens, horses with *S. fayeri* infection were seronegative using the standard immunoblot test without *S. cruzi* absorption (Cutler et al., 2001). Thus, the elimination of antibodies that cross-react with *S. cruzi* from equine serum prior to standard immunoblot testing serves no readily apparent purpose.

The negative control equine sera used to develop the new test method were from horses native to India and Germany (Rossano et al., 2000). A total of 63 horses, six positive and 57 negative were examined. Many of these serum samples may contain antibodies to equine *Sarcocystis* spp. that may be unique to the Eastern hemisphere. These species were not utilized to develop the standard immunoblot test, which may help explain the divergent results. Test comparison was confounded by the lack of appropriate negative controls. In 1995, 300 serum samples were tested from wild horses in Utah, an area with canids (*S. fayeri* present), but not opossums. Although anti-*Sarcocystis* reactivity was observed routinely, only one sample tested positive (D. Granstrom, unpublished observation, 1995). This type of true negative serum sample from North American horses would provide an interesting comparison of test performance.

The 30 and 16 kDa proteins used for interpretation of the modified test by Rossano et al. (2000) are not *S. neurona*-specific; serum from horses infected with *S. fayeri* recognize both proteins. When bovine blocking serum was not used, 95% of the negative control equine sera reacted with the 30 kDa protein and 81% reacted with the 16 kDa protein. In addition, 19% of the samples continued to react to the 30 kDa protein and 11% to the 16 kDa protein when bovine serum was used. Prior to blocking with bovine serum, 16–67% of the negative control equine sera reacted with at least one of the four proteins tentatively identified by Rossano et al. (2000) as those used to interpret the standard test. The original test was not designed to test horses infected with *Sarcocystis* spp. from the Eastern hemisphere. When control blots using bovine antiserum only were developed using labeled anti-bovine IgG, the 30 and 16 kDa proteins and those proteins tentatively identified as *S. neurona*-specific failed to react. The mechanism responsible for blocking antigen access to equine antibodies under these conditions is difficult to appreciate.

Various studies have attempted to assess the validity of immunoblot test results using postmortem examination as the gold standard (Daft et al., 1997; Sleiman et al., 1997; Mohammed and Swaney, 1999). A number of important factors must be considered when attempting to evaluate reports of this type. These include: contamination of spinal fluid with blood, quality of CSF, clinical course in the horse, and the time interval between CSF collection and euthanasia. Since the blood–brain barrier deteriorates rapidly, postmortem CSF must be collected immediately. Histopathologic examination of a limited number of tissue sections from a few sites along the cervical spinal cord is not adequate because only a few parasites are seen in most cases of EPM (Boy et al., 1990). Evaluations must be standardized and consistently applied to find minute, multifocal lesions and to reliably

account for the possibility of more than one etiology. *S. neurona* infection anywhere in the CNS may result in a detectable amount of intrathecal antibody production.

The results of immunoblot tests on serum and CSF from 295 horses euthanatized due to neurologic disease were compared to postmortem diagnoses during 1991–1996 (Granstrom, 1997). Serum samples were available from 191 horses, CSF from 254 horses, and both samples were available from 88 horses. CSF samples with visible blood contamination were not tested. Approximately 40% of the horses had histologic lesions compatible with EPM. Other diagnoses included cervical vertebral malformation (CVM), trauma, hepatic encephalopathy, viral encephalitis, equine degenerative myeloencephalopathy (EDM), leucoencephalomalacia, CNS abscessation, epilepsy, cauda equina syndrome, neurotoxicity, lymphosarcoma, botulism, guttural pouch mycosis, and aberrant parasite migration.

Sensitivity and specificity of the immunoblot test on CSF were both 89%. Sensitivity represents the number of horses that tested CSF positive of all horses diagnosed with EPM at postmortem, i.e., the number of true positive tests divided by the number of true positives plus false negatives. Specificity reflects the number of horses that tested CSF negative from all the horses that did not have EPM at postmortem, i.e., the number of true negative tests divided by the number of true negatives plus false positives. Therefore, the immunoblot test should detect *S. neurona* antibodies in the CSF of 9 of 10 horses with EPM from a population of horses with neurologic signs. Interestingly, three of the horses that tested false negative had been ill for less than 2 weeks. In most cases, the incubation period of EPM appears to be sufficient to permit production of detectable amounts of specific antibody. Acutely ill horses that initially test negative should be re-tested. Apparently, a small percentage of horses fail to produce a detectable antibody response.

Approximately 1 in 10 horses with neurologic signs may test positive for *S. neurona* antibodies in CSF even though another neurologic disease is responsible. If the blood–brain barrier is compromised, antibodies from the bloodstream may leak into the CSF, resulting in a false positive test. False positive CSF test results were caused by CVM, viral encephalitis, trauma, EDM, leucoencephalomalacia, and CNS abscessation. However, the most common cause of false positive CSF results is believed to be blood contamination at the time of collection.

The positive predictive value (PPV) calculated from CSF test results was 85%. The PPV represents the proportion of truly diseased horses that actually tested positive, divided by the number of true positive tests plus false positives (Cohen and McKay, 1997). Negative predictive value (NPV) represents the proportion of non-diseased horses among all those that tested negative, i.e., the total number of horses without EPM at postmortem divided by the number of true negative tests plus false negatives. The NPV among this population was 92%. It is important to note that test sensitivity and specificity remain constant when used among various populations. However, the PPV and NPV for any diagnostic test vary dramatically, dependent upon the prevalence of the disease among the population tested. The PPV is severely impacted when used in a population with low disease incidence, i.e., the normal horse population, unless test sensitivity and specificity are 100%. Any test with an 89% sensitivity and specificity would yield a PPV of 8% in a population with 1% incidence of disease. In contrast, the NPV is high when prevalence is low.

The PPV was calculated using CSF from horses that were euthanized due to neurologic disease. It cannot be used to interpret CSF test results from horses without neurologic signs.

In addition to the mathematical reasons cited above, too little is known about the pathobiology of *S. neurona* in the horse to adequately interpret CSF test results from clinically normal horses.

The results of seroprevalence studies conducted among various horse populations represent *S. neurona* exposure only (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Tillotson et al., 1999). Although many horses are exposed, the incidence of clinical disease remains low. Test sensitivity and specificity for serum samples from the postmortem study support this observation (Granstrom, 1997). Test sensitivity on available serum samples was 89%, but specificity was only 71%. Specificity was low because 30% of the horses with other neurologic diseases were also exposed to *S. neurona*. Similarly, the PPV for serum was 72%. The NPV for serum was 88%, indicating the value of a negative blood test among neurologic and normal horses. When interpreted appropriately, immunoblot testing of equine serum and CSF samples provides veterinarians with valuable information regarding exposure to *S. neurona*. Exposure to *S. fayeri* and similar organisms is differentiated. Nonetheless, continued improvement for clinical use is warranted.

Antibodies produced against other parasites that may cross-react with *S. neurona* by immunoblot analysis have not been determined. Marsh et al. (1996b) indicated cross-reactivity between *N. hughesi* and *S. neurona* based on a horse that died due to neosporosis. Careful evaluation of the report indicates this conclusion was confounded. Given the high exposure rate to *S. neurona*, it seems more likely the horse was exposed to *S. neurona* as well as *N. hughesi*. Although CSF albumin concentration and AQ values were within normal limits, the relative lack of sensitivity of these measures indicate that anti-*S. neurona* antibodies in CSF may have been serum-derived. In addition, *S. neurona* and *N. hughesi* may have been present in the CNS concurrently. A controlled experimental infection done in an isolation facility using horses with no anti-protozoal antibodies in serum or CSF would be required to accurately evaluate this hypothesis. Interestingly, a parallel study in the same report, using rabbit antiserum prepared against *N. caninum* failed to demonstrate cross-reactivity with any of the specific proteins used for standard testing. Lindsay and Dubey (2001) have recently described a direct agglutination test to detect antibodies to *S. neurona* in experimentally infected animals. This test needs to be further evaluated in horses and compared to the Western blot for sensitivity and specificity.

5.4. PCR

PCR testing of equine CSF also provides information regarding the presence of *S. neurona* DNA in the CNS (Fenger et al., 1994; Marsh et al., 1996b). The sensitivity of the PCR test appears to be much lower than initially estimated. Apparently, intact merozoites rarely enter CSF and free parasite DNA is destroyed rapidly by enzymatic action (Marsh et al., 1996a). Nonetheless, parasite DNA has been detected in CSF samples that tested negative for antibodies to *S. neurona*. The PCR test may be a useful adjunct for the diagnosis of EPM in selected cases.

5.5. Pathology

When present, the gross lesions of EPM are confined to the CNS (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974). Acute lesions consist of multifocal randomly

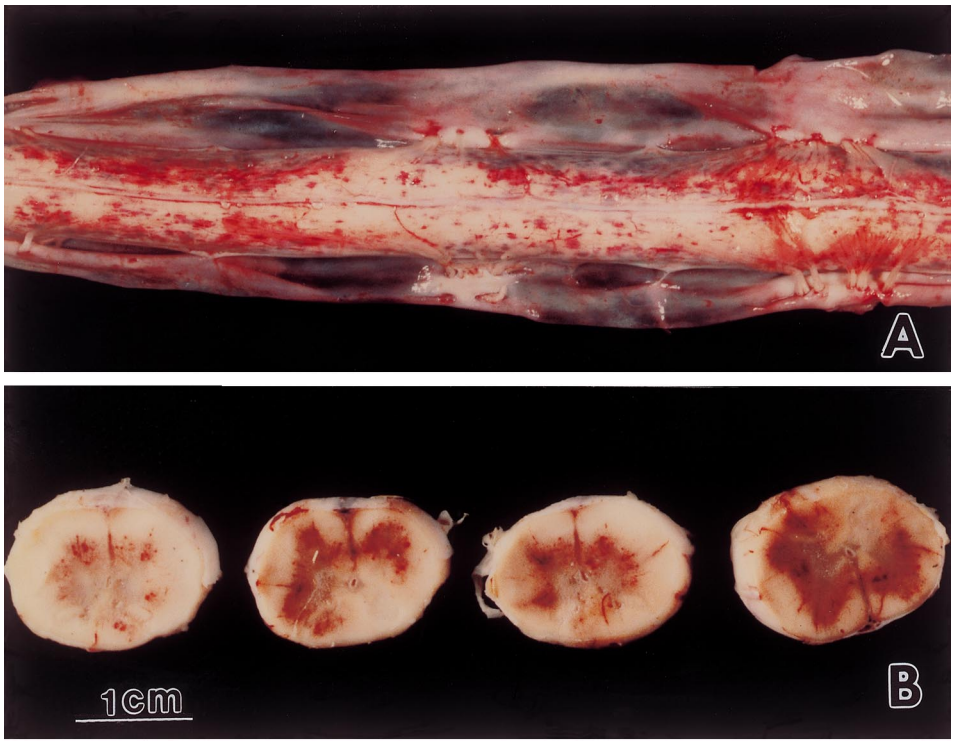

Fig. 10. Acute hemorrhagic lesions in the spinal cords of two horses with EPM. (A) The dura has been cut longitudinally and reflected away from the cord which shows presence of numerous multifocal to coalescing acute hemorrhages. (B) Cross-sections of a spinal cord of a horse with EPM. Variable sized acute hemorrhages are present predominantly within the gray matter of the cord. (Photos courtesy of Dr. A.N. Hamir, USDA, ARS, Ames, IA).

distributed foci of hemorrhages (Fig. 10), whereas subacute and chronic lesions show areas of discoloration ranging from pale to dark tan areas (Fig. 3A) and foci of malacia, respectively. Although the brainstem is more often involved than other areas of the brain, the lesions are more frequently seen in the spinal cord. In rare cases, lesions may be present in both the brain and the spinal cord of a horse. Microscopically, the predominant lesion is multifocal to coalescing areas of hemorrhage, nonsuppurative inflammation, and small foci of necrosis (Cusick et al., 1974; Beech and Dodd, 1974; Mayhew and de Lahunta, 1978; Clark et al., 1981; Dorr et al., 1984; Boy et al., 1990; Hamir et al., 1993; Dubey et al., 1974, 1999b, 2001d). Perivascular cuffing by mononuclear cells is evident in some of the affected areas, particularly in the meninges (Fig. 11A). The inflammatory response is highly variable (Fig. 11) and may consist of infiltrates of a mixture of lymphocytes, neutrophils, eosinophils, multinucleate giant cells, and gitter cells. Chronic lesions are uncommon because most of the affected horses die or are euthanized during the acute stage of the disease or the affected horses may have received anti-protozoal and anti-inflammatory therapies prior to death (Boy et al., 1990).

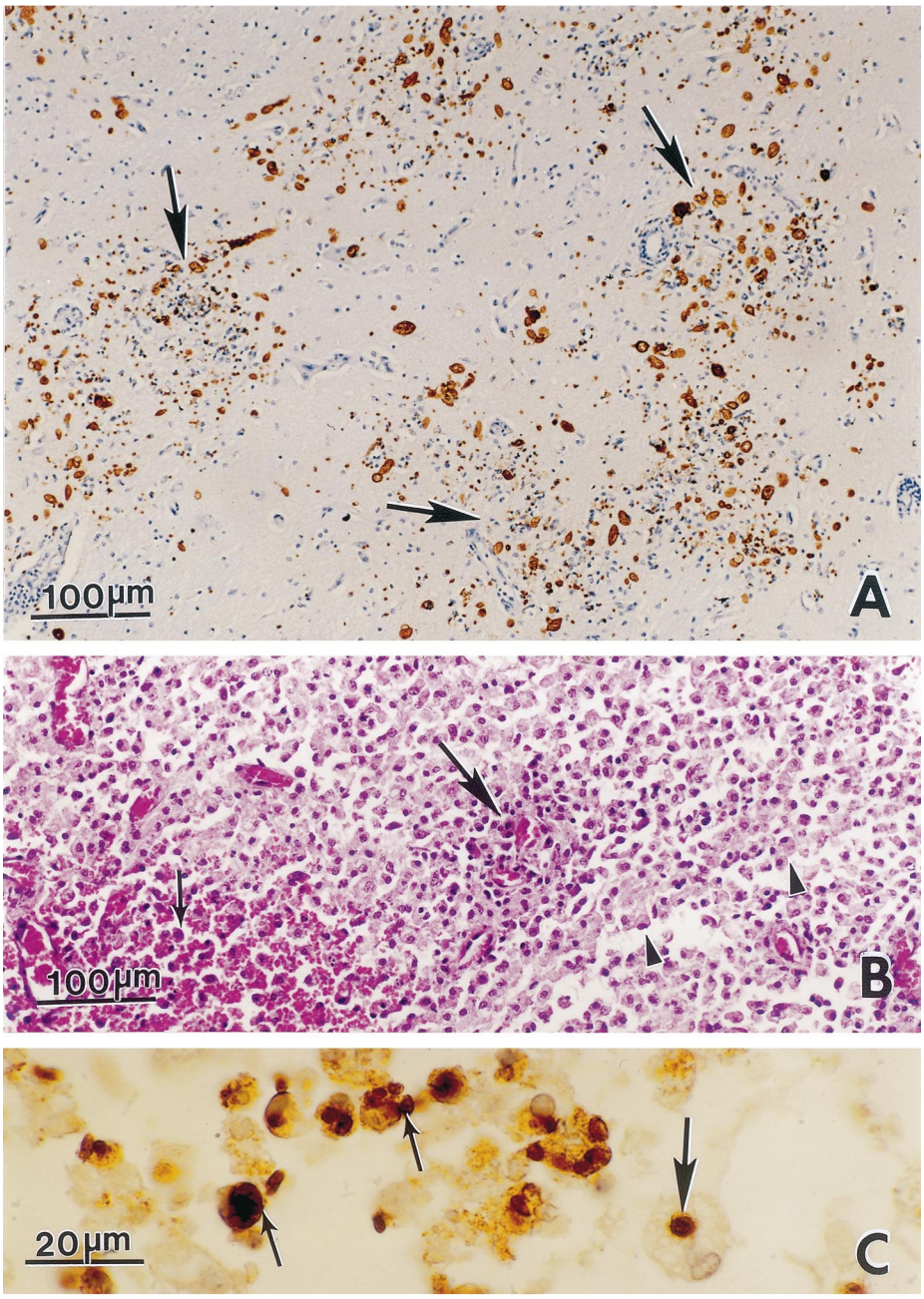

Fig. 11. Lesions and *S. neurona* in CNS of horses. (A) Brain with multifocal areas of perivascular cuffs (arrows). Immunohistochemical stain with anti-*S. neurona* serum. All brown spots are organisms. (B) Brain with focal vasculitis (large arrow), hemorrhage (small arrow) and macrophages (arrowheads). H&E. (C). Spinal cord with merozoites in macrophages (large arrow) and in unidentified inflammatory cells (small arrows). Immunohistochemical stain with anti-*S. neurona* serum.

The numbers of *S. neurona* stages present are often few and difficult to locate in routine histological sections stained with hematoxylin and eosin (H&E). There was no association between the presence of organisms in H&E stained sections and the therapeutic administration of corticosteroids in a group of 82 cases of EPM (Boy et al., 1990). However, in a suspect EPM horse given dexamethasone, Dubey et al. (1974) found numerous organisms in the gray matter throughout the thoracic spinal cord (Fig. 3B–E); *S. neurona* was named from the organism seen in this horse (Dubey et al., 1991a).

Developmental stages of *S. neurona* are more easily seen if organisms are present in neurons rather than in inflammatory cells (Fig. 3). The types of host cells which are infected are not definitively known except that schizonts and merozoites are found in neurons, giant cells, neutrophils, and in macrophages (Figs. 3 and 11). The etiology of lesions is not fully known. The number of organisms are usually few even in extensive lesions suggesting that cytokines/metabolites may be associated with lesions. The cause of hemmorrhagic lesions is unexplained because parasites are usually extravascular.

5.6. Microscopic differential diagnosis

The organisms considered in the differential diagnosis of *S. neurona* infection are: *T. gondii*, *Neospora* species, *Sarcocystis* species, and microsporidian parasites. Although *S. neurona* was once thought to be *T. gondii*, there is no documented case of acute toxoplasmosis in horses (see Dubey and Beattie, 1988; Dubey et al., 1999a). Moreover, *T. gondii* is structurally and antigenically distinct from *S. neurona*; *T. gondii* divides by endodyogeny, whereas *S. neurona* divides by endopolygeny to make many merozoites per schizont. Therefore, there is no immature stage of *T. gondii*, whereas immature schizonts of *S. neurona* which contain a large lobed or dividing nucleus can be observed prior to merozoite production. This is helpful in distinguishing the two parasites in sections.

Rarely, *Neospora* organisms (*N. hughesi* and possibly *N. caninum*) are associated with EPM. Clinical neosporosis has been reported in seven horses. One was an aborted fetus (Dubey and Porterfield, 1990), one was a month-old foal (Lindsay et al., 1996), four were aged mares with complicating conditions (Marsh et al., 1996a,b; Daft et al., 1996; Hamir et al., 1998; Cheadle et al., 1999) and one was a 10-year-old horse with possible immunodeficiency (Gray et al., 1996). *Neospora* organisms (Fig. 12D and E) structurally resemble *T. gondii* and are distinct from *S. neurona* for the same reasons *T. gondii* is distinct (Fig. 12). *N. caninum* tissue cysts are distinct from *T. gondii* because the cyst wall of *N. caninum* is 1–4 μm thick, whereas tissue cysts of *T. gondii* have thin (<1 μm) cyst walls.

S. neurona is rarely seen in vascular endothelium, whereas *S. fayeri* and other *Sarcocystis* species schizonts occur in vascular endothelium (Fayer and Dubey, 1982; Dubey et al., 1989; Hamir et al., 1997). Hamir et al. (1997) reported a case of *Sarcocystis* encephalitis with EPM-like lesions; the parasite was present in vascular endothelium (Fig. 12A and B) and was considered different from *S. neurona*. So far, there is no report of *S. neurona* infection in extra-neural tissues of horses; however, Hamir and Dubey (2001) recently diagnosed *S. neurona* in the myocardium of raccoons, and studies in immunodeficient mice suggest that other organs may be involved. Davis et al. (1999) described hepatitis associated with a *Sarcocystis*-like organism (Fig. 12C). However, the parasite was not *S. neurona* because

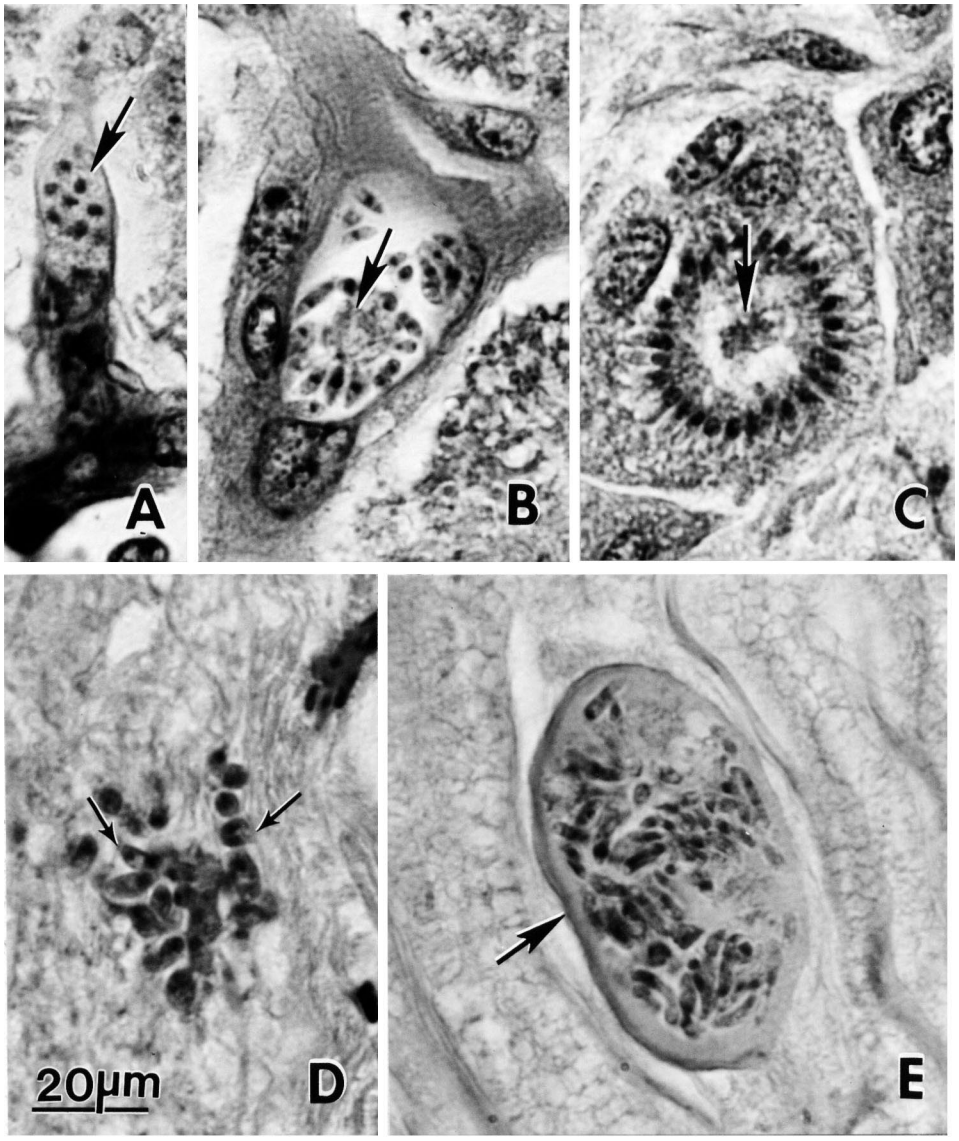

Fig. 12. *Sarcocystis* and *Neospora* species in tissues of horses. H&E. Bar = 20 μm and applies to all figures. (A) and (B) Intravascular schizonts (arrows) of *Sarcocystis* sp. in the brain of the horse reported by Hamir et al. (1997). The schizont in (A) is immature (arrow). Schizont in (B) has a residual body (arrow) and some merozoites have separated from the main mass. (C) *Sarcocystis* sp. schizont with a residual body (arrow) in a hepatocyte in the liver of the horse reported by Davis et al. (1999). (D) *Neospora* sp. tachyzoites in spinal cord of the horse reported by Hamir et al. (1998). Note tachyzoites dividing into two zoites (arrows). (E) A thick-walled tissue cyst (arrows) of *Neospora* sp. in sciatic nerve of the horse reported by Daft et al. (1996) (slide courtesy of Dr. B.M. Daft, University of California, Davis, CA).

it was structurally and antigenically distinct. Ultrastructurally, *Sarcocystis* schizonts are distinct from other parasites because the merozoites lack rhoptries (see Fig. 6).

5.7. Immunohistochemistry

Immunohistochemical tests can distinguish *S. neurona* from other organisms (Lindsay and Dubey, 1989; Dubey and Hamir, 2000). It is important to use a *S. neurona*-specific serum for immunohistochemistry because other sera with antibodies to *Sarcocystis* (e.g. *S. cruzi*) do cross-react with *S. neurona* antigens (Granstrom et al., 1991; Hamir et al., 1993). At present, there are no *S. neurona*-specific monoclonal antibodies useful for diagnosis.

6. Treatment

Treatment of horses suspected to have EPM should be done as quickly as possible after clinical signs of the disease are recognized. Treatment appears to result in successful recovery in 70–75% of the affected horses, although, without postmortem confirmation it is somewhat difficult to know the true meaning of this comment.

For many years treatment has been confined to the use of dihydrofolate reductase inihibitors such as sulfonamides and pyrimethamine (Mayhew et al., 1976; MacKay et al., 1992). In fact, the traditional therapy used to treat horses with EPM has been a prolonged course (up to 12 weeks or longer) of pyrimethamine and sulfadiazine. Usual treatment involves the use of sulfadiazine at a dose of 20 mg/kg per os, once or twice a day. In addition, affected horses should be placed on pyrimethamine (Daraprim, Burroughs Wellcome). The dosage is 1.0 mg/kg given once a day per os for 120 days or longer. Duration of treatment may be longer if the CSF remains positive and/or the horse continues to demonstrate clinical signs of neurological disease. Complications of anemia and/or leukopenia have been observed, especially when the dose of pyrimethamine is doubled, and in some horses diarrhea has occurred.

A determination to discontinue treatment is based on either significant improvement of the clinical signs or the horse returning to normal and Western blot testing of CSF returning to negative. The combination of sulfadiazine and pyrimethamine results in a sequential blockade of folic acid metabolism. The specific concentration of pyrimethamine required to achieve an anti-protozoal level for *S. neurona* is not known. However, it is known that *T. gondii* and *N. caninum* are susceptible at 1 μg/ml of pyrimethamine alone and 0.1 μg/ml when combined with sulfadiazine (Lindsay and Dubey, 1999).

Diclazuril (Clinacox, Scherring-Plough Animal Health, Union, New Jersey, USA), a coccidiostat, is an alternative treatment for horses which have failed to respond to the traditional therapy or in horses which have developed complications (Granstrom et al., 1997; Cohen, 1998; Dirikolu et al., 1999). The drug is absorbed quickly and has been found in serum 1 h after feeding to horses (Dirikolu et al., 1999). It is in the benzeneacetonitrile group and has been used as a prophylactic agent against coccidiosis in poultry and has been used experimentally in the treatment of similar problems in rabbits. It has anti-*S. neurona* activity in cell cultures infected with *S. neurona* (Lindsay and Dubey, 2000). Recently, diclazuril was found to have anti-*S. neurona* activity in KO mice fed lethal doses of sporocysts. Mice

fed diclazuril in rodent pelleted feed (50 parts per million) starting 5 days before or 7 days after feeding *S. neurona* sporocysts and continuing therapy for a month killed *S. neurona* stages in the mice. Therapy was less effective when diclazuril was given 12 days or more after feeding sporocysts; unmedicated mice developed neurologic signs were euthanized between 22 and 30 days after feeding sporocysts. These results indicate that diclazuril can kill the early stages of *S. neurona* and may be useful as a prophylactic agent against *S. neurona* infections in horses (Dubey et al., 2001b).

Toltrazuril (Baycox 5% suspension; Bayer, Canada) is an anti-coccidial drug used in several species. The mechanism of action is to disrupt intracellular pathways important in energy metabolism as well as cell division. This drug has potential efficacy for the treatment of EPM (Furr, 2000). This drug appears to have good oral absorption and fairly long elimination time (48–72 h). The drug has good lipid solubility and is well absorbed into CSF. In horses given toltrazuril at 5 mg/kg daily for 10 days, plasma levels of toltrazuril were 20 mcg/ml with a mean CSF concentration of 160 mcg/ml (Furr and Kennedy, 2000). The use of this drug has not been shown to result in any complications nor have elevations of serum chemistry values or changes in complete blood counts been observed. A metabolite, ponazuril (toltrazuril sulfone), was recently utilized in a multicenter treatment study (seven sites) involving 100 horses. The drug appeared to show very favorable clinical results and is currently under review by the FDA for marketing in US. Ponazuril has in vitro activity against *S. neurona* (Lindsay et al., 2000a).

One additional drug for EPM is nitazoxanide (NTZ, Navigator, Romark Laboratories). It has broad spectrum activity against bacteria, protozoa and helminths. The drug is effective in killing *S. neurona* in cell culture (Lindsay et al., 1998) and has been recently tested in a clinical field trial for the treatment of horses with EPM. The drug appears to have good oral absorption, although the concentration found in CSF following six clinical doses of 50 mg/kg was nil. The safety studies indicated that at a 2× dose for 1 week horses appeared lethargic and at 4× dosing horses showed illness and one died. An efficacy study of 70 horses showed 63% of the horses improved 1 grade or more or became negative on Western blot testing of CSF. Dosing at this time is suggested to begin at 25 mg/kg daily per os for the first 7 days followed by 50 mg/kg for a total treatment time of 30 days (McClure and Palma, 1999).

When horses are treated using dihydrofolate reductase inhibitors, folic acid deficiency and anemia may be side effects of the treatment (Toribio et al., 1998). In human beings, megaloblastic anemia is a common side effect of treatment with pyrimethamine. To combat this problem, we recommend frequent evaluation of the complete blood count while the horse is on treatment, and if evidence of anemia is identified, the treatment should be discontinued and diet should be supplemented with folinic acid. In human beings, folinic acid (5-formyl-THF), a form of bioactive tetrahydrofolate, is used to combat anemia. The protozoa cannot utilize preformed folate that allows use of folic acid. Folic acid administration has two potential problems in the horse. The first is that the drug is poorly absorbed from the intestinal tract and the second is that to convert the folate to the active form of tetrahydofolate requires dihydrofolate reductase which is being inhibited by the treatment. Treatment of pregnant mares with sulfonamides, pyrimethamine, folic acid, and vitamin E can cause congenital deformities (Toribro et al., 1998), and there is a suggestion that EPM treatment can affect the breeding performance of stallions (Bedford and McDonnell, 1999).

Supplemental or ancillary treatments such as immune stimulants have been suggested for possible assistance in the treatment of horses with EPM. Corticosteroids should be avoided in horses suspected to have EPM as this may worsen the clinical signs. However, when faced with a rapidly deteriorating case, one or two doses of corticosteroids can be administered to help to reduce the inflammation and allow time for the anti-protozoal medications to work. If clinical signs persist, therapy may be re-evaluated every 30 days.

7. Epidemiology

Knowledge regarding the epidemiology of EPM has been slowly evolving. The first major report about EPM was generated from 10 centers throughout US and Canada involving 364 histologically confirmed cases (Fayer et al., 1990). The greatest percentage of cases were in horses 4 years of age (61.8%) or less. Thoroughbreds, Standardbreds and Quarter Horses made up the greatest percentage of the cases; however, there did not appear to be a breed predilection (Fayer et al., 1990). Another study involved 82 horses in Pennsylvania with EPM by Boy et al. (1990) who found the risk was higher in male and Standardbred horses (Boy et al., 1990). Unfortunately, neither of these studies had control populations for comparison, and the study by Fayer et al. (1990) compiled data supplied by different centers without any verification. Since those initial reports, numerous cases of the disease have been diagnosed throughout North, Central, and South America. Cases of EPM seen in Europe, South Africa, and Asia were in horses that had been imported from the Western hemisphere (Mayhew and Greiner, 1986; Ronen, 1992; Lam et al., 1999).

Early rates of exposure to *S. neurona* were between 10 and 30% when small studies were performed at farms in central Kentucky and Ohio (Moore et al., 1995). Development of a Western blot test for antibody to *S. neurona* in the early 1990s led to larger studies of exposure rates to this organism in Pennsylvania, Oregon, Ohio (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997) and recently in Colorado (Tillotson et al., 1999). The rate of exposure in both Oregon and Pennsylvania were approximately 45% with both studies finding an effect of increasing prevalence with increasing age. The rate of exposure was higher in Ohio (53.6%), but an age effect was found in that study also (Saville et al., 1997). In both Oregon and Ohio, there were location differences in exposure rates in different areas throughout each state. These location differences may be related to climate differences, which may have an effect on parasite transmission.

In the study from Colorado, antibodies to *S. neurona* were found in 33.6% of 608 sera from various equids submitted to a laboratory in Colorado. Prevalence increased with age. In 1–5-year-old horses, prevalence was 26.0% versus 37% in 10-year-old horses. Seroprevalence was lowest during the colder months (Tillotson et al., 1999). Although other equids have been exposed to *S. neurona*, there is only one report of clinical EPM in a pony (Dubey and Miller, 1986; Dubey and Hamir, 2000) and one in a zebra (Marsh et al., 2001). Antibodies to *S. neurona* were found in 24 of 49 (49%) ponies, 11 of 18 (61.1%) donkeys and mules from Ohio (Saville et al., 1997) and 66.6% of ponies and other nonhorse-equids from Colorado (Tillotson et al., 1999). These results suggest that nonhorse equids are resistant to clinical EPM. Recently, antibodies to *S. neurona* have been reported from horses in Brazil

and Argentina. The seroprevalence rates reported were 35.6% in Brazil and 35.5% from Argentina (Dubey et al., 1999a,d). Results from these five studies are comparable because immunoblots were performed in one laboratory.

Limited studies have been performed to measure the prevalence of exposure to *N. caninum or N. hughesi*, both potential causative agents of EPM. Five studies have reported exposure rate of horses to *N. caninum* (Dubey et al., 1999a,c,d; Cheadle et al., 1999; Pronost et al., 1999). A larger study was performed using samples from horse slaughterhouses in Texas and Nebraska (Dubey et al., 1999c). The prevalence of *N. caninum* antibodies in the US study was 23.3% by the *Neospora* agglutination test (NAT); however, no information was available regarding age, breed, gender or origin. Pronost et al. (1999) reported NAT antibodies in 55% of 67 horses from France and also reported the presence of *N. caninum* DNA by PCR in an aborted equine fetus. The studies from Brazil and Argentina did not find detectable antibodies to *N. caninum*; however, small numbers of horses were included in both studies, 101 and 76 horses, respectively (Dubey et al., 1999a,b). It would appear that approximately 50% of the horses in some areas are exposed to *S. neurona*. Based on limited numbers of studies, exposure rates to *N. caninum* are somewhat less than half of the number exposed to *S. neurona*. Perhaps this lower rate of exposure to *N. caninum* is related to the low numbers of oocysts that are excreted in dog feces (McAllister et al., 1998; Lindsay et al., 1999). Performance of larger studies may help to corroborate these findings. Regardless, these studies have provided good information to help better understand the epidemiology of EPM.

No planned studies have been performed to examine the prevalence of clinical EPM in US. Prevalence of EPM in horses was estimated at 0.5–1% of the horse population (Granstrom, 1997). This estimate was based on the number of submissions to Dr. Granstrom's laboratory at the University of Kentucky when it was the only laboratory performing the diagnostic test for *S. neurona* antibody (Granstrom et al., 1993). Little information is available regarding the prevalence of EPM demonstrated to be caused by *N. caninum* or *N. hughesi*, but seven cases of neosporosis have been reported throughout US (Dubey and Porterfield, 1990; Daft et al., 1996; Gray et al., 1996; Lindsay et al., 1996; Marsh et al., 1996a,b; Hamir et al., 1998; Cheadle et al., 1999). Of the seven cases of neosporosis reported, only five of the cases exhibited neurologic signs.

Original reports of EPM cases suggested that the disease is sporadic and more than one case is seldom seen at a particular farm (Mayhew et al., 1976; MacKay et al., 1992). However, clusters of cases have occurred in a few instances, which would suggest that all of the risk factors necessary for disease were at those facilities (Granstrom et al., 1992; Fenger et al., 1997b). Clustering of cases was corroborated by recent findings from a controlled investigation at the Ohio State University (OSU). In the OSU study, if EPM had been diagnosed at a farm prior to diagnosis in one of the cases in the study, the risk for EPM was >2.5 times higher than if EPM had never been diagnosed before (Saville et al., 2000b). EPM has been reported in siblings (Traver et al., 1978).

Trends such as age, breed, season or other findings associated with development of clinical cases of EPM reported in previously published case series had not been reported from controlled investigations. A study from OSU compared horses diagnosed with EPM to two control populations. One control population was horses admitted to the hospital for diseases in other organ systems (non-neurologic controls) and the other control group

included horses diagnosed with other neurologic diseases (neurologic controls) (Saville et al., 2000b). When comparing horses diagnosed with EPM to non-neurologic controls, young horses (1–5 years old) and older horses (>13 years) had a higher risk of developing EPM than horses <1 year and 6–13 years of age. Cases of EPM and neurologic controls exhibited similar use and signalment which may have precluded an effect of age in that comparison series. Compared to the winter, the risk for EPM was three times higher in spring and summer and six times higher in the fall (Saville et al., 2000b). Since climactic factors such as freezing days appear to affect exposure rates, there is no reason to suspect that it may not affect rates of disease. Timing of annual athletic competitions is often in the fall of the year; therefore, transport stress may also contribute to the seasonal effect (Saville et al., 2000b). There was a 2.5 times higher risk of EPM if opossums were observed on the property where horses resided compared to never seeing an opossum (Saville et al., 2000b). Protection of feed from wildlife access was associated with a one-third lower risk of EPM. The risk for EPM was 50% lower if a creek or river was on the premises, compared to premises without a creek or river (Saville et al., 2000b). The OSU study suggests the presence of opossums and their access to horse feed are important risk factors; however, if an alternate habitat for wildlife such as a creek or river is available, the risk may be reduced. If wooded areas were present on the premises where horses resided, there was twice the risk for disease. This finding of wooded areas on the property may be related to the natural habitat of the definitive host. A strong dose response relationship between health events and the risk for EPM was also demonstrated in the OSU study (Saville et al., 2000b). Risk increased as the time at which the health event occurred prior to admission to the OSU veterinary hospital up to a period of 6 months. Health events such as aging, exercise, transport, injury, surgery, or parturition may lead to immune suppression with subsequent development of clinical signs of EPM. Race horses and show horses had the highest risk for EPM compared to breeding and pleasure horses when occupation of the horse was examined. This occupational risk may be related to age, intensity of exercise or perhaps economic value. Improvement in clinical signs of horses diagnosed with EPM was lower in breeding and pleasure horses compared to racehorses and show horses (Saville et al., 2000a). Breeding and pleasure horses usually are older and often receive no or little exercise. Aging has been associated with increased risk for infectious disease in other species. In addition, moderate exercise enhances immune function in horses. Therefore this difference in improvement may be related to a combination of exercise intensity and age and their potentially immunosuppressive effects. If horses diagnosed with EPM were treated, there was a 10 times higher probability that EPM cases would improve compared to untreated horses. However, this estimate may be somewhat biased as the most severely affected horses are the least likely to be treated for extended periods of time. Two factors, severity of clinical signs and improvement in clinical signs, were associated with the likelihood of survival (Saville et al., 2000a). If horses diagnosed with EPM exhibited moderate or severe clinical signs, they were less likely to survive than horses with mild neurologic deficits. Horses that demonstrated an improvement in clinical signs were 50 times more likely to survive than horses whose clinical signs did not improve (Saville et al., 2000a). This is the first study that examined the factors that are associated with development of clinical signs of EPM. This study may have determined factors that may be manipulated to prevent EPM. Monitoring of horses during high risk periods may also help to prevent some cases of the disease.

The distribution of EPM follows the distribution of opossums. In North America, *D. virginiana* occurs widely except on Prince Edward Island in Canada. The number of sporocysts shed by opossums has not been determined, although millions of sporocysts of undetermined species may be present in opossum intestines (Dubey, 2000, 2001). The exact prevalence of *S. neurona* in opossums is unknown. The main reason for this is that there is no simple method to identify *S. neurona* in opossum feces. Opossums are hosts for three named species of *Sarcocystis*: *S. neurona*, *S. falcatula*, *S. speeri*, and probably other unnamed species (Dubey and Lindsay, 1999; Tanhauser et al., 1999; Dubey et al., 1998, 2000a, 2001d). Because the complete life cycles of these parasites are not known, there is no morphometric method to identify sporocysts of different species. Three methods have been used to determine the prevalence of *S. neurona* in opossums: biologic, molecular, and in vitro cultivation. *S. speeri* and *S. neurona* sporocysts are infective to gamma interferon gene KO mice and produce encephalitis, whereas *S. falcatula* does not infect mice (Marsh et al., 1997a,b; Dubey and Lindsay, 1998). However, *S. neurona* can be immunohistochemically distinguished from *S. speeri* (Dubey and Lindsay, 1999). *S. falcatula* and *S. falcatula*-like organisms are infective to budgerigars, whereas *S. speeri* and *S. neurona* do not infect budgerigars (Marsh et al., 1997a; Dubey and Lindsay, 1998, 1999). Using a bioassay method, intestinal scrapings from 44 road killed opossums were examined for *Sarcocystis* species. The prevalence was *S. falcatula* (47.7%), *S. neurona* (18.1%), and *S. speeri* (18.1%). *S. neurona* alone was found in one opossum, *S. speeri* alone in one opossum, and mixed *Sarcocystis* species were present in 21 opossums (Dubey, 2000). In another planned survey, *S. neurona* was found in 19 of 72 opossums trapped in rural Mississippi (Dubey et al., 2001a).

Using genetic markers, Tanhauser et al. (1999) identified *S. neurona* in five of nine opossums from Florida. Murphy and Mansfield (1999) found *Sarcocystis* sporocysts in seven of 27 opossums from Michigan, and they successfully grew *Sarcocystis* spp. schizonts in cell culture from all seven opossums using sporocysts as the inoculum. Although species were not identified, the isolates were thought to be *S. neurona*. Based on these surveys, the prevalence of *S. neurona* in opossums is very high. Because *Sarcocystis* sporocysts are trapped in the intestinal lamina propria (Dubey et al., 1989), sporocysts are released into the intestinal lumen for months, and while only a few sporocysts may be present in feces, millions may be trapped in the intestine.

8. Economics

According to American Horse Council, the equine industry is worth $112 billion in the US economy. In 1995 exports of horse carcasses were estimated at $67.5 million and exports of live horses were estimated at $285 million (US Department of Agriculture (USDA), Animal Plant Health Inspection Service (APHIS) Report, June 1995). A recent survey was conducted to identify needs of the US horse industry and to determine the priorities for the National Animal Health Monitoring System (NAHMS) equine 1998 study. This survey involved 2599 respondents, 76% of which were horse owners, the remainder being veterinarians and other representatives of the horse industry. Of the infectious diseases listed, EPM was 24% and ranked first (USDA, APHIS Report, May 1997). The number of

cases of EPM diagnosed in horses with neurological signs at the OSU has increased from 24.9% in 1992 to 50% in 1996.

The cost of diagnostic neurological evaluation at OSU is approximately $456 per horse. Treatment of horses for EPM can be expensive, especially since most affected horses need to be treated for a period of 120–150 days, and sometimes longer. Based on prices at one hospital, the cost of treatment is approximately $200.00 per month for a 450 kg horse. Re-evaluation of the horse at 30–60-day intervals and a subsequent spinal tap at 90–120 days following the initiation of treatment adds to the cost. Treating horses with toltrazuril is estimated at $1200 (5 mg/kg) to $2400 (10 mg/kg) for 30 days and diclazuril at $770 for 30 days. The American Horse Council estimates that there are 6.6 million horses in US. Clinical EPM occurs in 0.5–1.0% of horses (Granstrom, 1997). Using the estimated prevalence rates of clinical EPM the direct costs for diagnosis and treatment of EPM in US range from $55.4 to $110.8 million per year. This figure does not include such indirect costs as decreased performance time, loss of stake payments, transport costs, death or euthanasia.

9. Laboratory maintenance, animal models, protective immunity, and vaccines development

9.1. Experimental animal models

At present, there is no fully reproducible method to induce clinical *S. neurona* infections in a large animal. Fenger et al. (1997a) in Kentucky fed sporocysts from opossums to *S. neurona*-negative horses. The horses fed sporocysts developed neurologic signs, antibodies to *S. neurona* in blood and CSF, lesions in CNS, but *S. neurona* was neither demonstrated histologically nor by bioassay in cell culture. Similar results were obtained by two other groups of researchers in Florida and Ohio. Cutler et al. (2001) in Florida fed horses an inoculum considered to contain only *S. neurona* sporocysts. It was concluded that the clinical illness was more severe in horses given multiple doses of sporocysts and in those given corticosteroids. In the third study conducted in Ohio, EPM was more severe in horses that were stressed by transportation. Administration of corticosteroids did not affect the clinical outcome (Saville et al., 2001). Actually, the disease was less severe in horses given corticosteroids. The fact that clinical signs and lesions were induced in all three studies, but the parasite was not demonstrated in equine tissues suggests that most horses may be able to eradicate *S. neurona* from their tissues. Horses injected with *S. neurona* merozoites directly in the CSF developed antibodies to *S. neurona*, but remained clinically normal (Lindsay et al., 2000c).

Marsh et al. (1997b) discovered that nude mice were susceptible to parenteral inoculation with culture-derived merozoites of *S. neurona*. Nude mice inoculated intraperitoneally with 10,000 or more merozoites developed encephalitis associated with *S. neurona*; C57/Black, BALB/c, and SCID mice were not susceptible to *S. neurona* infection (Marsh et al., 1997b).

Dubey and Lindsay (1998) reported that gamma interferon gene KO mice fed sporocysts from opossums developed encephalitis, and *S. neurona* was demonstrable in lesions. The migration and development of *S. neurona* was recently studied in KO mice fed sporocysts from a naturally infected opossum (Dubey, 2001). Mice were killed 1–36 days after feeding sporocysts (DAFS). Zoites were seen in intestinal epithelial cells 1 DAFS (Fig. 13A),

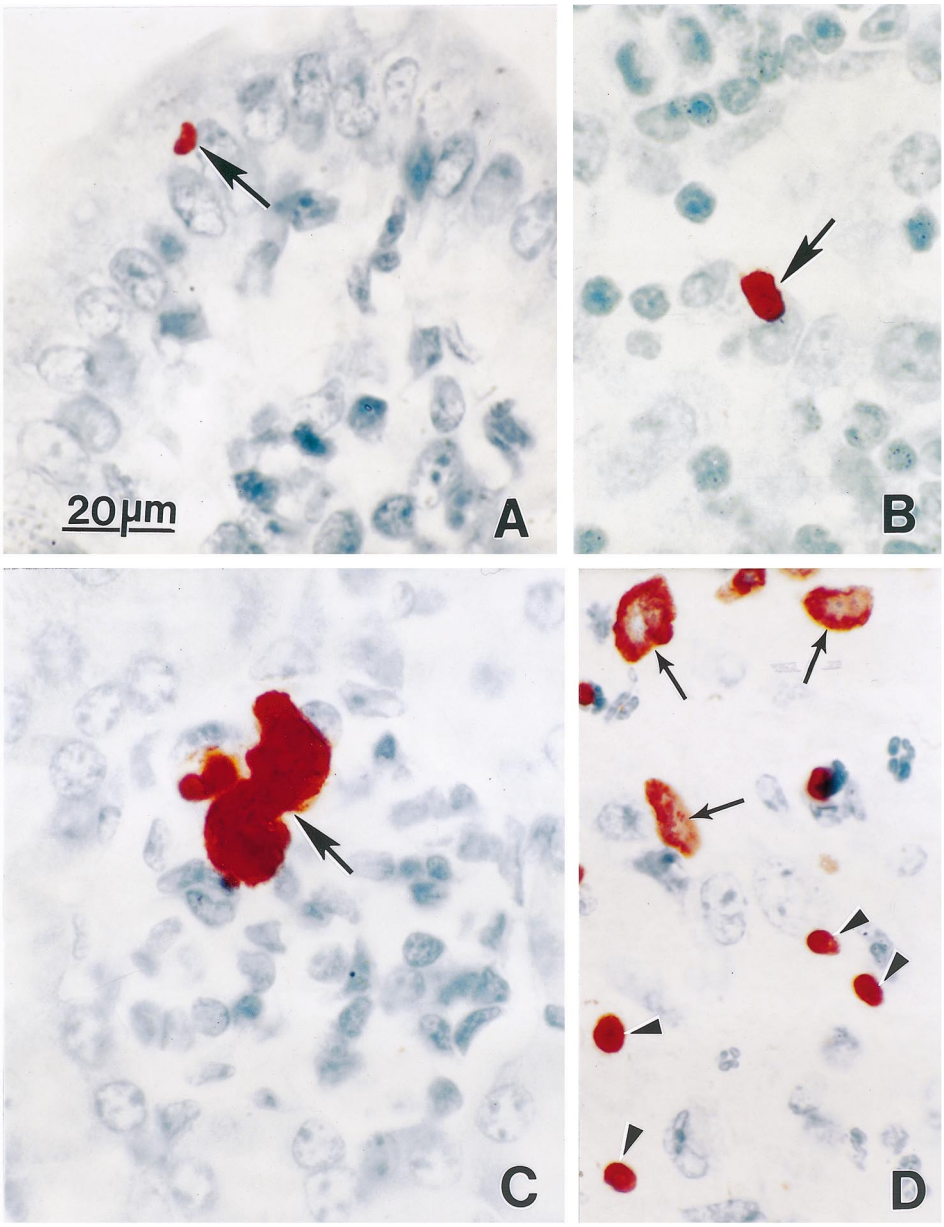

Fig. 13. Stages of *S. neurona* sections of tissues of KO mice 1–13 days after feeding sporocysts (DAFS) from isolate SN15-OP (Dubey, 2000). Immunohistochemical stain with anti-*S. neurona* serum. All figures are at the same magnification. (A) A zoite (arrow) in villar epithelial cell of small intestine. 1 DAFS. (B) A zoite (arrow) in a mesenteric lymph node. 2 DAFS. (C) Schizonts (arrow) in a renal glomerulus. 7 DAFS. (D) Schizonts (arrows) and merozoites (arrowheads) in brain. 13 DAFS.

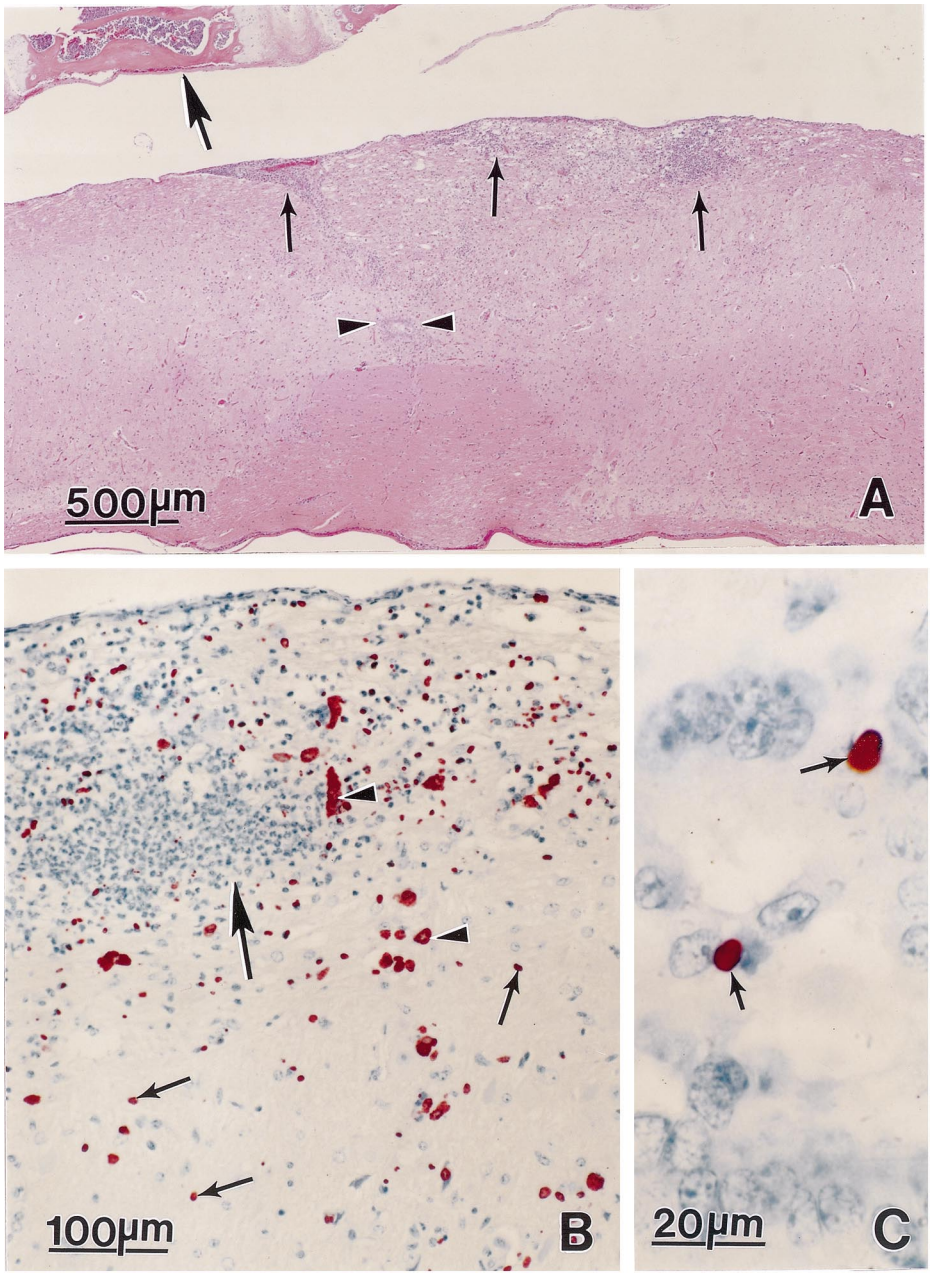

Fig. 14. Lesions and *S. neurona* in cervical spinal cord of a KO mouse 22 days after feeding sporocysts from a naturally infected opossum. (A) Longitudinal section in situ with spinal vertebrae (large arrow). Note lesions (small arrows) in white matter and in the spinal canal (arrowheads). H&E. (B) A microabscess (large arrow) and numerous *S. neurona* schizonts (arrowheads) and merozoites (small arrows). Immunohistochemical stain with anti-*S. neurona* serum. (C) Spinal canal with merozoites in ependymal epithelial cells (arrows). Immunohistochemical stain with anti-*S. neurona* serum.

in mesenteric lymph nodes 2 DAFS (Fig. 13B), in the liver 4 DAFS (Fig. 13C) and in several visceral tissues at 6–8 DAFS (Fig. 13C). *S. neurona* was isolated by bioassay in KO mice from the brains of mice 7 DAFS and was detected histologically at 9 DAFS. Most multiplication of *S. neurona* occurred in CNS, 11 DAFS. These data indicate that *S. neurona* multiplies to a limited extent in visceral tissues during the first 11 DAFS, and thereafter it is confined mainly to the brain and spinal cord of mice (Fig. 14). In mice killed 20 DAFS, *S. neurona* was found occasionally in lungs, heart, and the eye (Dubey, 2001). These data on migration and development of *S. neurona* will be useful to target chemotherapy against specific stages of the parasite in KO mice. KO mice fed *S. neurona* sporocysts are usually not sick until 14 days after feeding sporocysts, irrespective of the dose or stage administered.

The number of *S. neurona* merozoites needed to produce clinical infections in KO or nude mice has not been determined. Nude mice inoculated intraperitoneally with 10,000 or more merozoites developed neurologic signs 30 or more days after injection (Marsh et al., 1997b; Dubey and Lindsay, 1998).

9.2. In vitro cultivation of S. neurona

S. neurona has been cultivated directly from the spinal cords of EPM horses with neurologic signs (Table 2). Three (SN1, SN6, SN7) of the isolates listed in Table 2 were obtained

Table 2
Characteristics of isolates of *S. neurona* obtained from spinal cords of horses with neurologic signs[a]

Isolate designation	Location of horse	Year of cultivation in cell culture	Cell type, initial cultivation	Pathogenicity to mice	References
SN-1	New York	1990	M617	Unknown	Dubey et al. (1991a,b)
SN-2	California	1990	M617	Pathogenic	Davis et al. (1991a,b), Dubey and Lindsay (1998)
SN-3	Panama	1991	M617	Unknown	Granstrom et al. (1992), Liang et al. (1998)
SN-4	California	1991	M617	Unknown	Davis et al. (1991); unpublished, mentioned by Granstrom et al. (1994)
SN-5	Kentucky	1992	M617	Unknown	Granstrom et al. (1994)
UCD-1	California	1993	M617	Pathogenic	Marsh et al. (1996, 1997)
UCD-2,3	California	1995			
SN-6	Oregon	1998	M617, equine spleen	Pathogenic	Dubey et al. (1999b)
SN-7	Oregon	1998	Equine spleen	Pathogenic	Dubey et al. (2001d)
SN-MU1	Missouri	1999	Equine dermal	Unknown	Marsh et al. (2001)
M1H1	Michigan	1997	Equine dermal	Unknown	Mansfield et al. (2001)
M1H2	Michigan	1997	Equine dermal	Unknown	Mansfield et al. (2001)
M1H3	Michigan	1997	Equine dermal	Unknown	Mansfield et al. (2001)
M1H4	Michigan	1997	Equine dermal	Unknown	Mansfield et al. (2001)
M1H5	Michigan	1997	Equine dermal	Unknown	Mansfield et al. (2001)
M1H6	Michigan	1998	Equine dermal	Unknown	Mansfield et al. (2001)
M1H7	Michigan	1998	Equine dermal	Unknown	Mansfield et al. (2001)
M1H8	Michigan	1998	Equine dermal	Unknown	Mansfield et al. (2001)

[a] Modified from Dubey et al. (1999b).

Table 3
Details of *S. neurona* isolates obtained from KO mice fed sporocysts from naturally infected opossums (*D. virginiana*) from US

Isolate designation	Opossum No. and reference	Source	Cell line[c]	Date inoculated in cell culture
SN8-OP	8030[a]	Manheim, PA	ED	6-22-98
SN9-OP	8047[a]	Zoo, LA	ED	7-24-98
SN10-OP	8064[a]	College Park, MD	M617	10-29-98
SN11-OP	8071[a]	College Park, MD	M617	11-6-99
SN12-OP	8095[a]	College Park, MD	ED	7-15-98
SN13-OP	8105[a]	College Park, MD	ED	11-3-98
SN14-OP	8129[a]	Zoo, LA	M617	10-31-98
SN15-OP	9108[a]	Montros, VA	M617	7-14-99
SN16-OP	1[b]	Mississippi	M617	5-14-99
SN17-OP	2[b]	Mississippi	M617	5-17-99
SN18-OP	3[b]	Mississippi	BT	12-27-99
SN19-OP	4[b]	Mississippi	EK	8-7-00
SN20-OP	6[b]	Mississippi	EK	8-7-00
SN21-OP	7[b]	Mississippi	EK	7-28-00
SN22-OP	8[b]	Mississippi	BT	2-24-00
SN23-OP	9[b]	Mississippi	EK	7-28-00
SN24-OP	11[b]	Mississippi	EK	4-4-00
SN25-OP	12[b]	Mississippi	BT	4-3-00
SN26-OP	13[b]	Mississippi	EK	4-4-00
SN27-OP	14[b]	Mississippi	BT	4-12-00
SN28-OP	16[b]	Mississippi	BT	4-11-00
SN29-OP	17[b]	Mississippi	BT	4-8-00
SN30-OP	18[b]	Mississippi	EK	4-20-00
SN31-OP	21[b]	Mississippi	EK	5-10-00
SN32-OP	22[b]	Mississippi	BT	5-22-00
SN33-OP	23[b]	Mississippi	EK	6-5-00
SN34-OP	24[b]	Mississippi	EK	5-30-00

[a] Dubey (2000).
[b] Dubey et al. (2001a).
[c] BT: bovine turbinate, ED: equine dermal, EK: equine kidney, M617: bovine monocytes.

from horses that were given corticosteroids to increase parasitization. Recently, *S. neurona* (isolate designated SN-0T1) was isolated in BT and CV-1 cells inoculated with brain homogenate of a Southern sea otter with neurologic signs (Lindsay et al., 2000b). *S. neurona* was isolated from the brains of KO mice fed sporocysts from feces of 34 naturally infected opossums (*D. virginiana*) from US (Table 3). Two isolates (SN35-OP and SN36-OP) were obtained from the South American opossum (*D. albiventris*) from Brazil (Dubey et al., 2001c). All these 36 isolates of *S. neurona* from opossums were pathogenic to KO mice (Dubey, 2000; Dubey et al., 2001a,c).

Numerous cell lines, including bovine monocytes, equine kidney cells, equine dermal cells, cardiopulmonary endothelial cells, deer testes, vero cells, CV-1 cells, rat myoblasts, and bovine turbinate cells, can support the growth of *S. neurona* (Davis et al., 1991a,b; Granstrom et al., 1992; Marsh et al., 1996a,b; Lindsay et al., 1999; Speer et al., 2000; Mansfield et al., 2001). In most EPM horses the number of *S. neurona* in CNS tissue is low.

Therefore, culture flasks seeded with CNS homogenates should be incubated for at least 2 months because some strains are slow to adapt in cell cultures. Once established, *S. neurona* can complete schizogonic development in 3 days (Davis et al., 1991a,b; Lindsay et al., 1999). The number of merozoites produced may vary with cells used, culture conditions, and the strains of *S. neurona* (Speer et al., 2000). Development of the SN2 isolate of *S. neurona* was examined by Lindsay et al. (1999). Merozoites were usually located next to the host cell nucleus and occasionally appeared to be in the host cell nucleus. Division was asynchronous resulting in cells containing structurally different organisms. Intracellular merozoites measuring 7.9 × 2.9 μm were the only stages seen 1 day post-inoculation (PI). Merozoites increased in size and became developing schizonts (Fig. 4A). Merozoites with a lobed nucleus were observed 2 days PI (Fig. 4B). These developing schizonts were 12.9 × 5.4 μm. Developing and mature schizonts were present 3 days PI. The developing schizonts were 24.6 × 12.9 μm. Merozoites in mature schizonts had a posteriorly located nucleus and were 6.7 × 1.5 μm 3 days PI. Merozoites budded from the schizont surface prior to cytokenesis. A residual body was often visible in mature schizonts observed 3–6 days PI. Developing schizonts present 4 days PI were 27.6 × 15.2 μm. Merozoites in mature schizonts were 5.8 × 1.7 μm 4 days PI (Fig. 4B). Developing schizonts present 5 days PI were 26.2 × 14.2 μm. Merozoites in mature schizonts were 5.6 × 1.7 μm 5 days PI. Developing schizonts present 6 days PI were 24.2 × 13.7 μm. Merozoites in mature schizonts were 5.8 × 1.6 μm 6 days PI (Fig. 4B).

S. neurona has also been isolated from sporocysts from the intestines of opossums either directly in cell cultures (Murphy and Mansfield, 1999) or by feeding sporocysts to KO mice and then recovering *S. neurona* in cell cultures from mouse brains (Dubey and Lindsay, 1998). Using this procedure, *S. neurona* was isolated from 8 of 44 opossums (Table 3). In KO mice fed sporocysts, *S. neurona* parasitizes the brain during the 2nd week after feeding sporocysts, and the hind brain is more heavily parasitized than the cerebrum (Dubey, 2001). Therefore, the hind brain should be included in the homogenate seeded into cell cultures.

Murphy and Mansfield (1999) reported a simple technique of isolating *Sarcocystis* species from sporocysts from opossums. Sporocysts were sterilized with sodium hypochlorite, washed, crushed between a coverslip and glass slide, and mechanically released sporozoites were inoculated into cell cultures. Dubey and Lindsay (1998) reported reviving the SN-2 strain of *S. neurona* 8 years after storage in liquid nitrogen; the revived strain induced fatal infection in KO mice.

9.3. Protective immunity and vaccine development

Preliminary studies indicate that some strains may lose pathogenicity for KO mice by prolonged in vitro cultivation (Dubey et al., 2001d). Therefore, *S. neurona* should be cryopreserved between experiments.

Nothing is known at present regarding the nature of protective immunity to *S. neurona* infection. Results of experiments with interferon-gamma gene KO mice indicate that certain cytokines mediate immunity to *S. neurona* infection. Liang et al. (1998) reported antibodies to two surface proteins Sn14 and Sn16 (14 and 16 KDa) of *S. neurona*, have an inhibitory effect on penetration of merozoites in cultured cells. Thus, antibodies may play a role in

mediation of immunity, although *S. neurona* is an intracellular organism. Horses injected with killed *S. neurona* merozoites can develop antibodies to *S. neurona* and this has stimulated research into development of a possible *S. neurona* vaccine for horses.

10. Perspective

Much needs to be learned of immunity to *S. neurona* in horses before an effective vaccine can be developed against EPM. The pathogenesis of EPM in the horse is unknown. Why only a small proportion of infected horses develop clinical infections is unknown. Why it is difficult to induce clinical EPM in horses remains an enigma? Are there other unknown definitive hosts for *S. neurona*? The natural intermediate host/hosts are unknown. Questions to many of these answers will not be known unless a reproducible equine model for *S. neurona* is developed.

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