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SHORT COMMUNICATION

J. B. Dame · T. J. Cutler · S. Tanhauser
S. Ellison · E. C. Greiner · R. J. MacKay

Equine protozoal myeloencephalitis: mystery wrapped in enigma

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Introduction

Equine protozoal myeloencephalitis (EPM) is the most important infectious neurologic disease of horses in North America (Fenger 1997; MacKay 1997). The disease has emerged from first recognition in the 1960s to almost epidemic proportions in the 1990s. A wide variety of clinical signs are possible including paresis, cranial nerve deficits, vestibular disease and seizures. However, the majority of affected horses exhibit milder signs, such as gait abnormalities (consisting of weakness and ataxia) and localized muscle atrophy. Frequently, the disease is chronic and slowly progressive and, consequently, efforts to make a diagnosis are often delayed. The causative organism was first isolated from an affected horse's spinal cord in 1991 and was named *Sarcocystis neurona* (Dubey et al. 1991). However, it is often not possible to conclusively demonstrate the organism in the central nervous system of diseased horses, and the diagnosis remains presumptive. Immunohistochemistry allows definitive diagnosis in some additional cases (Hamir et al. 1993). To further complicate diagnosis, a *Neospora* sp. has been identified as an additional, albeit rare, cause of EPM from the western U.S (Daft et al. 1996; Marsh et al. 1996, 1998; Hamir et al. 1998). Seroprevalence data suggest that infection with *S. neurona* is becoming more widespread. Earlier reports had suggested seroprevalence was $<20\%$ (Granstrom 1993a). In 1997, surveys of horses from three states across the United States revealed that $\sim 50\%$ of horses had serum antibodies reactive with *S. neurona* antigens on immunoblot, and that seroprevalence increased with age (Bentz et al. 1997; Blythe et al. 1997; Saville et al. 1997). Work from our laboratory now suggests that these data may already underestimate seroprevalence (T.J. Cutler, and R.J. MacKay, unpublished observations). Disease and seroconversion have also been reported from Canada and South America (MacKay 1997). Clearly, many horses in the Americas have been exposed to *S. neurona*. While the complete life-cycle is not known, the opossum (*Didelphis virginiana*) has been shown to be a natural definitive host (Fenger et al. 1995, 1997). Recent studies have shown that the opossum also sheds at least three other *Sarcocystis* spp.(Tanhauser et al., in press). Efforts are now being directed to life-cycle studies, to understand the interrelationships among these *Sarcocystis* spp., and to develop a mouse model of EPM (MacKay 1997; Dubey and Lindsay 1998; Dubey et al. 1998; Tanhauser et al., 1999). Much of the work in our laboratory has been focused on inducing EPM in naïve horses. Ultimately, a horse-challenge method will be necessary for evaluation of pharmaceuticals, for vaccine development and to fulfill Koch's postulates.

In this paper we review the evidence that *S. neurona* causes EPM, the evidence that the opossum is the definitive host for multiple *Sarcocystis* spp. parasites, and the results of several experimental challenges in horses.

Sarcocystis neurona is the principal agent of EPM

Spinal cord lesions consistent with EPM have been recognized in horses residing in North and South America for more than 25 years (Rooney et al. 1970; Macruz et al. 1975). Many sarcocystid protozoans may infect the central nervous system of animals, including *Toxoplasma*, *Sarcocystis*, and *Neospora* spp. Initial descriptions of EPM suggested that *Toxoplasma gondii* was the cause, but subsequently it was determined that the agent was actually a *Sarcocystis* sp. (Simpson and Mayhew 1980). Critical microanatomic characteristics that support this conclusion include division by endopolygeny and the absence of both a parasitophorous vacuole and rhoptries. Further, the organism shared

J. B. Dame (✉) · T. J. Cutler · S. Tanhauser · S. Ellison
E. C. Greiner · R. J. MacKay
College of Veterinary Medicine,
University of Florida, Gainesville, FL 32611-0880, USA

antigenic cross reactivity with *S. cruzi* (Hamir et al. 1993) but not *Toxoplasma* or *Neospora* spp. (Bowman et al. 1992).

In 1991 an organism was successfully isolated in continuous culture from the spinal cord of a horse with EPM, and was named *S. neurona* (Davis et al. 1991). A number of primary isolates have subsequently been obtained in several laboratories by culturing spinal cord lesions. The various isolates are reported to be similar to each other antigenically, but a considerable amount of work remains to compare these isolates biologically and at protein and nucleic acid levels. A retrospective immunohistochemical study of suspected EPM cases identified reactive antigen in spinal cord sections using both *S. neurona* (51% of cases) and *S. cruzi* (67%) antisera (Hamir et al. 1993). These results suggest that most EPM cases are due to *S. neurona*. The 33% of cases that failed to react with *S. cruzi* antisera may indicate either that antigen was absent in the sections examined or that a different agent was responsible. Indeed, other causes of EPM have been reported, but appear considerably less common than *S. neurona* infection. *Neospora* spp. have been reported in the CNS of three horses with EPM (Daft et al. 1996; Marsh et al. 1996; Hamir et al. 1998), and there is evidence that one, if not all, of these parasites may differ sufficiently from *Neospora caninum* to be a different species, recently named *N. hughesi* (Marsh et al. 1998). Further, an unidentified *S. neurona*-like organism dividing by endopolygeny, but not staining with either *S. neurona* or *S. cruzi* antisera, was identified in a horse from Oregon (Hamir et al. 1997).

Ante-mortem diagnosis of EPM presents an even more difficult challenge. Several large surveys from Ohio, Oregon, and Pennsylvania used horse sera drawn for regulatory testing to estimate seroprevalence, using an immunoblot for antibodies directed against *S. neurona* antigen (Bentz et al. 1997; Blythe et al. 1997; Saville et al. 1997). Seroprevalence was estimated at between 45% and 55%. In comparison, it has been estimated that only 1–2% of infected horses actually develop the disease EPM (MacKay 1997). For these reasons, intrathecal antibodies detected using undiluted CSF in the immunoblot assay are considered to have a much greater specificity for EPM. Published data are limited, but it is claimed that the immunoblot test on CSF is both highly sensitive and specific (each > 90%) when compared to horses confirmed to have EPM using histopathology (MacKay 1997). The positive and negative predictive values of these test results vary with the prevalence of EPM in the test population of horses. Despite some limitations, the immunoblot test using CSF from horses with clinical signs of neurologic disease is the best ante-mortem diagnostic available for EPM (Granstrom 1993a, b).

The opossum is the definitive host of *Sarcocystis neurona* (and several other *Sarcocystis* spp.)

Sarcocystis spp. have an obligatory heteroxenous life-cycle. A predator or scavenger is the definitive host of the parasite, and is infected after ingesting muscle of prey (intermediate host) containing the sarcocyst stage of the parasite. The sarcocyst is the only stage that is infectious to the definitive host and that can be used for morphological identification of species. It may be found in skeletal muscle, enteric smooth muscle, or myocardium. In the definitive host, the life-cycle occurs completely within the enteric mucosa. Sporulated sporocysts are shed in feces and are immediately infectious to the intermediate host, which becomes systemically infected and eventually completes the life-cycle by forming sarcocysts. The horse is considered an aberrant intermediate host of *S. neurona* because only the pre-cystic stage, the merozoite, has been found in equine tissues. Because *S. neurona* sarcocysts have yet to be identified in a natural intermediate host, it is possible that this organism has previously been named and is considered a benign parasite of that intermediate host. In addition, the challenge of additional candidate definitive hosts may be necessary to explain the occurrence of EPM where opossums are rare. This is also precluded by the lack of characterized *S. neurona* sarcocysts.

The opossum was identified as a potential definitive host of *S. neurona* by comparing the sequence of the nuclear small subunit ribosomal RNA (SSURNA) gene of *S. neurona*, isolated from a horse, with sporocysts from the feces of wild-caught animals (Fenger et al. 1994). The advent of a PCR-based method to identify *S. neurona* in any stage of its life-cycle made identification of intermediate host(s) a realistic possibility. Additionally, once a candidate intermediate host species had been identified, it could be challenged with characterized *S. neurona* sporocysts isolated from opossums. We hypothesized that the intermediate host of *S. neurona*

might already have been identified and previously named *S. falcatula*, a parasite of opossums and brown-headed cowbirds and grackles (Box et al. 1984). Using a similar approach to Fenger's (Fenger et al. 1994, 1995), we examined segments of SSURNA that contained >75% of the species-specific markers distinguishing *S. neurona* from *S. muris* and found that, within experimental error, the SSURNA gene sequences of *S. neurona* and *S. falcatula* were identical. From this data, we concluded that the two species were synonymous pending biological confirmation (Dame et al. 1995).

To test that hypothesis, we infected opossums with *S. falcatula* sarcocysts, recovered sporocysts and challenged 5 horses with 10^6 sporocysts each. Horses did not develop clinical signs of EPM, nor did they seroconvert (Cutler and MacKay 1999). We expected antibody production to occur even if disease did not, because seroprevalence was so high in U.S. horses. However, a repeat challenge of horses yielded the same results (Cutler and MacKay 1999). In contrast to this, Fenger et al. challenged naïve horses with pooled, uncharacterized sporocysts from wild-caught opossums, and reported both seroconversion and neurologic disease (Fenger et al. 1997). In that study, however, the parasite was neither recovered by culture nor demonstrated by direct or immunohistochemical staining in the CNS of experimental animals. In addition, Fenger et al. (1997) were able to induce disease in birds with the inoculum given to the horses. The paradox is explained if *S. neurona* and *S. falcatula* are not synonymous but are both present in the inoculum used by Fenger et al., while only *S. falcatula* was present in our inoculum.

It became clear that a different approach was necessary to demonstrate that the organisms were different, and we returned to examining the genomes. Four RAPD-derived DNA fragments and the ITS-1 sequence from the ribosomal RNA transcription unit were used as the basis for molecular markers that could distinguish between *S. neurona* and *S. falcatula* (Tanhauser et al. 1999). The RAPD-derived DNA fragments were cloned from isolates of *S. neurona* and *S. falcatula* and were sequenced. Comparable regions from the two species were between 95.5% and 98.4% (mean 97.4%) identical. To rapidly differentiate between the organisms, restriction endonuclease enzymes were chosen that would selectively cut either the *S. neurona* or *S. falcatula* DNA segments, but not both. A pair of enzymes was selected for each molecular marker such that identification of the *Sarcocystis* spp. DNA present would be by positive criteria, i.e., cut by one or other enzyme but not both. Results correlated well with the full DNA sequence information available. The ITS-1 segment was also PCR-amplified and sequenced.

Using these molecular tools, isolates from approximately 40 wild-caught opossums have been categorized into 4 groups: *S. falcatula*, *S. neurona*, and two new types, which are presumed to be new species based on DNA sequences. One type is closely related to, but equally dissimilar from, *S. falcatula* and *S. neurona* while the other is sufficiently different genetically to all three that the RAPD-derived DNA markers cannot be amplified using our current primers.

Other studies in nude mice, γ-interferon knockout mice, and budgerigars have confirmed that *S. neurona* is biologically and antigenically distinct from *S. falcatula*, and that other *Sarcocystis* spp. are shed by opossums (Dubey and Lindsay 1998; Dubey et al. 1998). We conclude from these studies that the opossum is definitive host for at least four different *Sarcocystis* parasites including *S. neurona*.

Sarcocystis neurona causes EPM in experimentally challenged horses

In a pilot study, we successfully caused seroconversion in horses by challenging them with *S. neurona* sporocysts. Subsequently, with a revised challenge protocol, we demonstrated induction of clinical signs of EPM in other naïve horses. Challenged horses developed antibodies in both serum and CSF, and some developed clinical signs consistent with EPM, whereas contemporary environmental sentinels did not (T.J. Cutler and R.J. MacKay, unpublished observations). Lesions consistent with infection by *S. neurona* have been demonstrated in the spinal cords of challenged horses but, to date, protozoans have not been cultured or identified by immunohistochemical staining. Partial success with these trials has encouraged our continued efforts to establish the life-cycle of *S. neurona* in the laboratory and to establish a protocol for experimentally inducing EPM under controlled laboratory conditions.

The case for *S. neurona* being the major causative agent of EPM is nearly complete, but work remains to fulfill Koch's postulates. It will be necessary to isolate the organism from challenged horses (by culture of spinal cord or body fluids) and demonstrate that it is the same organism administered during challenge using our molecular tools. Clearly, a major goal is to identify the intermediate host of *S. neurona*. We are currently challenging candidate intermediate hosts, using molecularly characterized *S. neurona* sporocyst isolates from wild-caught opossums. Until it is possible to replicate the entire life-cycle of *S. neurona* under laboratory conditions, we are using pooled, characterized *S. neurona* sporocyst isolates to challenge naïve horses and other candidate hosts under controlled conditions.

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