Sarcocystis fayeri Associated Anti-Toxin in Serum from Horses with Neuromuscular Disease

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ABSTRACT

Equine protozoal myeloencephalitis (EPM) is a syndrome in which the inflammatory component of disease is an identifying criterion for histological diagnosis, but not addressed in clinical diagnosis or treatment. Two parasites, *Sarcocystis neurona* or *Neospora hughesi*, are generally believed to be the cause EPM in horses. Elucidating the nature of the immune response in EPM, determining how *S neurona* causes disease and whether organisms need to be present to cause pathologic changes and clinical signs remain the highest priority areas identified by scientists.[6] The purpose of this study was to detect *S fayeri* antitoxin in horses using a synthetic peptide representing a partial sequence of *S fayeri*-toxin, determine the prevalence of *S fayeri* antitoxin in horses with neuromuscular disease, and associate clinical disease to inflammation using serum C-reactive protein.

INTRODUCTION

Inflammation is a well-accepted cause of disease in microbial infections. However, equine protozoal myeloencephalitis (EPM) is a syndrome in which the inflammatory component of disease is an identifying criterion for histological diagnosis, but not addressed in clinical diagnosis or treatment. It is generally believed that two parasites, *Sarcocystis neurona* or *Neospora hughesi*, cause EPM in horses. Due to the extreme difficulty of locating *S neurona* in the central nervous system (CNS) tissues of diseased horses, a presumptive diagnosis is made based on characteristic inflammatory changes.[1,2,3,4,5] The presumption that parasites are the cause of disease fosters the general view that CNS antibodies, adjusted by a serum, CSF titer ratio, can support a diagnosis of EPM due to *S. neurona*. Because there is passive transfer of antibody across a healthy blood-brain barrier CSF, antibodies are not a definitive indicator of EPM.[6] Elucidating the nature of the immune response in EPM, determining how *S neurona* causes disease, and whether organisms need to be present to cause pathologic changes and clinical signs remain the highest priority areas identified by scientists.[6]

A significant omission in the studies of equine protozoal disease is the role of *Sarcocystis fayeri*. Two Sarcocystis species infect horses in the United States, *S fayeri* and *S neurona*. The pathogenicity of *Sarcocystis* that infect horses is variable. Infections can

Table 1. *Seroprevalence of partial peptide S. fayeri toxin (SFt) and S. neurona antibodies in horse serum. Sarocystis sp represents horses with both S. neurona and SFt antibodies.*

A. Multiple species infections indicated by *Sarcocystis sp* that was SFt and S. neurona		
	Clinically normal (%)	Neuromuscular disease (%)
Sarcocystis sp. + (n=94)	32 (87%)	62 (74%)
Sarcocystis sp. –	5 (13.5%)	22 (26%)
B. *Sarcocystis fayeri* seropositive indicated by SFt seropositive ELISA		
SFt +/*S. neurona*– (n=21)	8 (61.5%)	13 (37%)
SFt –/*S. neurona*–	5 (37.5%)	22 (63%)
C. *Sarcocystis neurona* seropositive indicated by rSAG 1, 5, 6 seropositive ELISA		
S. neurona +/SFt– (n=28)	8 (61.5%)	20 (48%)
S. neurona –/SFt–	5 (37.5%)	22 (52%)

be mild to severe.[7] *Sarcocystis fayeri* sarcocysts are found in skeletal muscle of clinically ill horses that show myositis, weight loss, abnormal gait, and weakness.[8]

Sarcocysts are also incidental findings in clinically normal horses. Therefore, *S fayeri* sarcocystosis is considered sub-clinical. In one study, horses with neuromuscular disease had a higher number of sarcocysts than control horses, leading the authors to speculate the assumption that *S fayeri* is an incidental finding may not be accurate for all cases.[7]

A common feature of sarcocystosis in intermediate hosts is cerebral infection.[9] Inflammation is often associated with degenerating host cells and not schizonts.[10,11] Toxins are also associated with asexual stages of Sarcocystis in intermediate hosts.[12,13,14,15] Not surprisingly, toxins are associated with *S fayeri* sarcocysts.[12,14] *Sarcocystis fayeri* toxins are available to the immune system of the intermediate host during schizogony or during cyst degeneration. Because toxins are associated with the asexual stages of the protozoa it is anticipated that *S fayeri* infected horses may develop circulating antitoxin.

A Sarcocystis toxin that induces neuromyopathy in horses may explain the discord between seroprevalence and *S neurona* EPM in horses. Some sarcocystis toxins are neurotoxic and can cause dose-dependent enterotoxicity in rabbits using an ileal loop test.[12,14] Serologic tests may not discriminate species of protozoa that infect horses.

Collective data from studies comparing SnSAG2, 4/3 ELISA, and IFAT (genus specific tests) indicate that serum titers do not provide an accurate diagnosis of EPM.1 The discussions concerning serodiagnosis of *S neurona* infections are not based on species-specific serotyping. For example, IFAT relies on genus specific antigens that may detect *S fayeri* antibodies.[16,17] Test positive animal disease is attributed to *S neurona* infections because it is believed *S fayeri* rarely causes neuromuscular disease. It is generally believed that *S fayeri* antibodies are not present in CSF. However, at least one horse with presumptive EPM was *S fayeri* CSF positive (data not shown).

Failing to distinguish infections by species will overestimate *S neurona* sarcocystosis in a population. A recently described *S fayeri* toxin is a candidate antigen that may allow serotyping and relate both parasites to neuromuscular disease. The purpose of this study was to detect *S fayeri* antitoxin in horses using a synthetic peptide representing a partial sequence of *S fayeri*-toxin, determine the prevalence of *S fayeri* antitoxin in horses with neuromuscular disease, and associate clinical disease to inflammation using serum C-reactive protein.

Table 2. *Horses with and without clinical signs were evaluated for serum C-reactive protein concentration.*

A. All Horses (N=121)		
	Clinically normal (%) (N=37)	Neuromuscular disease (%) (N=84)
CRP <17 (n=42)	19/37 (51.4%)	23/84 (27.4%)
CRP >16 (n=79)	18/37 (48.6%)	61/84 (72.6%)
B. *Sarcocystis* Positive (N=94)		
	Clinically normal (%) (N=32)	Neuromuscular disease (%) (N=62)
CRP <17 (n=34)	16/32 (50%)	18/62 (29%)
CRP >16 (n=60)	16/32 (50%)	44/62 (71%)
C. *S. fayeri* positive (N= 21)		
	Clinically normal (%) (N=8)	Neuromuscular disease (%) (N=13)
CRP <17(n=4)	1/8 (12.5%)	3/13 (23.1%)
CRP >16 (n=17)	7/8 (87.5%)	10/13 (76.9%)
D. *S. neurona* Positive (N=28)		
	Clinically normal (%) (N=8)	Neuromuscular disease (%) (N=20)
CRP <17(n=10)	3/8 (37.5%)	7/20 (35%)
CRP >16(n=18)	5/8 (62.5%)	13/20 (65%)

MATERIALS AND METHODS

S fayeri ELISA

A peptide representing the partial internal sequence of *S fayeri* toxin (RLDGVAALALEAHDLSDFET), SFt, was synthesized (United BioSystems).[12] Microtiter plates were coated with the synthetic peptide as antigen and used for indirect ELISA as previously described.[18] Antibodies against recombinant SAG 1, SAG 5, or SAG 6 did not bind the SFt (data not shown).

Clinical Samples

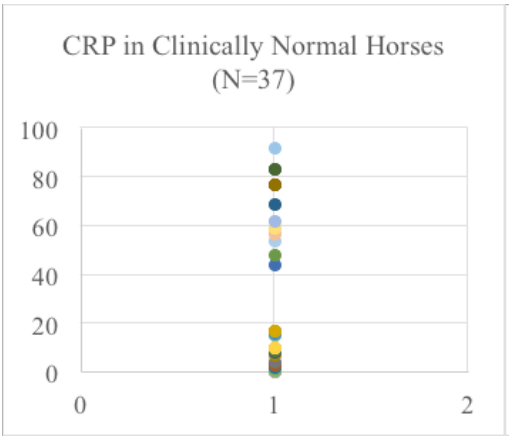
Serum samples were obtained from horses by licensed veterinarians that completed a neurological examination on each animal. Diseased horses had signs compatible with neuromuscular disease such as weakness, ataxia, or muscle atrophy. Serum was tested for antibodies (*S neurona* SAG 1, 5, 6, SFt) by ELISA and C-reactive protein concentration by ELISA (Genway, CA) as previously described.[19]

Serum C-reactive protein was determined in 248 normal, untreated horses. The median CRP was 17 µg/ml (data not shown). Reported values for infectious inflammatory disease in horses is 16 µg/ml (enteritis) and 19 µg/ml (pneumonia).[20] The cut off for a normal value was selected as 16 µg/ml in this study (Fig.1). The range was 0 µg/ml to 99 µg/ml. The relationship between neuromuscular disease and CRP was analyzed for significance using the Fisher's Exact Test.

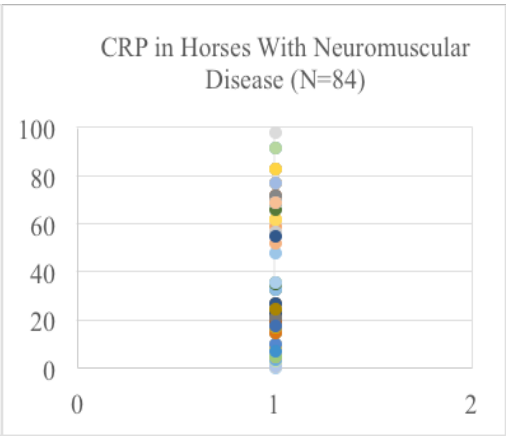
RESULTS

A survey of 42 clinically normal horses indicated that 24% were seropositive when tested for reaction to SFt (data not shown). The seroprevalence of Sarcocystis due to SFt and *S neurona* in clinically normal horses or horses with neuromuscular disease was compared (Table 1). Some clinically normal horses (87%) had antibodies to both Sarcocystis sp. Multiple infections in normal and diseased horses, indicated by Sarcocystis sp seropositive (normal, 87% and diseased, 74%), were more common than single species infections SFt (normal, 61.5% and diseased, 37%) or *S neurona* seropositive (normal, 61.5% and diseased, 48%). In animals with single Sarcocystis

Figure 1. *Serum C-reactive protein was determined in horses without (A) and with (B) neuromuscular disease. A cut-off for normal, CRP <16 µg/ml, was selected.*



A. Normal horses were equally likely to have a CRP concentration <17 µg/ml (normal) or >16 µg/ml (elevated).



B. Horses seropositive for Sarcocystis, irrespective of disease status, were likely to have an elevated CRP (>µg/ml).

infections (SFt n=21, S. neurona n=28), the presence of *S neurona* antibody (48%) was more often associated with neuromuscular disease in horses than horses seropositive for SFt (37%).

Acute phase C-reactive protein was evaluated in normal horses and those with neuromuscular disease, Figure 1 and Table 2. A cut-off for normal serum CRP concentration was less than 16 µg/ml for this study. An elevated CRP (72.6%) was detected in the majority of horses with clinical signs of neuromuscular disease, Table 2, section A. Significantly more horses with neuromuscular disesase had an elevated CRP when compared to normal horses (P = 0.0135) using Fisher's exact test, Table 2, section A. Seropositive horses, irrespective of clinical status or serotype, were likely to have an elevated CRP, section B, C, and D. Due to population sampling a difference in inflammation indicated by CRP concentration serotype was not made.

DISCUSSION

The incidence of *S fayeri* toxin antibodies in the population of normal horses in this study (23.8%) is similar to estimates of the prevalence of *S fayeri* infection in horses in the United States.[9] A peptide representing a partial protein associated with *S fayeri* sarcocysts (SFt) was used to detect the presence of serum antibodies against S fayeri-cyst toxin in horses. Single infections detected by SFt reactive antibodies or *S neurona* rSAG's was possible because serum positive for rSAG 1, 5, or 6 was not cross-reactive with SFt (data not shown).

Sarcocystis sp antibodies were found in diseased horses. However, *S neurona* seropositive sera (Table 1, section C) was more often associated with neuromuscular disease in horses than horses seropositive for SFt (Table 1, section B). This study showed that horses seropositive for both SFt and *S. neurona, Sarcocystis* sp., were more prevalent than single infections (Table 1). Horses with antibodies to both species of Sarcocystis were significantly more likely to show neuromuscular disease than those with single infections.

Serum C-reactive protein was detected in horses with and without neuromuscular disease, indicating the presence of inflammation in these animals. Significantly more horses with neuromuscular disease, defined by clinical signs (muscle atrophy or ataxia, and weakness), had an elevated CRP when compared to normal horses (P = 0.0135) using Fisher's exact test. The presence of

neuromuscular disease in some SFt seropositive horses may associate neuromuscular disease with *S fayeri* toxin antibodies.

C-reactive protein is not pathognomonic for disease. Cytokines stimulate CRP as an innate immune response to infections. An elevated CRP concentration in clinically normal, S fayeri-infected horses may indicate sub-clinical disease, as it does in other protozoal infections.[21] Clinically normal horses can harbor *S fayeri* sarcocysts in muscles.[7] It was determined that a dose dependent toxicity was associated with S fayeri-cyst protein in a rabbit model.[12] The rabbit ilial loop test was dose dependent, perhaps low levels of *S fayeri* toxin released from degenerating cysts in horses could be responsible for elevating serum CRP concentrations without initiating clinical disease. If innate immune responses that stimulate CRP are a result of *S fayeri* toxin, CRP may be a more sensitive measure of inflammation than clinical exam in seropositive horses.

Serum C-reactive protein may be a biomarker for inflammation in horses, horses seropositive for T gondii had higher levels of CRP than control horses.[21] Polyneuritis equi is a neuro-degenerative condition that was associated with circulating anti-myelin protein in horses.[22] C-reactive protein concentration has been suggested as a biomarker that is useful in evaluating horses with equine polyneuritis. [18,19] Serum CRP concentrations may be useful in clinical evaluation of Sarcocystis infections in horses with neuromuscular disease.[18,19] In this study, a cutoff of 16 µg/ml was used to determine a normal CRP value to analyze the data. This cutoff value, based on previous studies in horses, showed that most normal horses were negative and most horses with clinical disease were positive in this population. [20, 23]

Sarcocystis fayeri infections in horses are usually considered benign, however there is some evidence associating infection and neuromuscular disease.[7,8,9] Infections in horses proceed after sporocysts are ingested. The sporozoites undergo division and ultimately the asexual daughter cells (schizonts) enter the blood stream from the intestine. There is rapid clearance of the parasitemia and schizonts spread hematogenously to the viscera.[24] Schizonts are found in the intestine, liver/lung, and kidney for both equine-infecting Sarcocystis.[17,24] The terminally committed *S fayeri* schizont enters muscles where it encysts. Sarcocysts mature and degenerate with time in other species and most likely the process is similar in horses.[10,25,26] Cyst derived protozoa enter neighboring cells where they form new sarcocysts.[26]

Indirect fluorescent antibody tests (IFAT) detect schizonts in forming cysts in intermediate hosts. Because second-generation schizonts and developing cysts exhibit genus-specific antigens *S. neurona* and *S fayeri* are not distinguished by IFAT.[16] Serodetection of *S fayeri* toxin by ELISA may be more useful to detect *S fayeri* infections if species specific differences in Sarcocystis toxins are present.

Sarcocystine is a parasite toxin that was first associated with muscle cysts in 1897.[27] Clinical signs of neurotoxicity are demonstrated in rabbit models and include increased respiration, depression, and paralysis with eventual death.[12] Sarcocystine has been associated with Sarcocystis in humans, cattle, sheep, and other animals.[15,27,28,29] Most recently, *S fayeri* was associated with food poisoning in people.[12] Extensive characterization of the *S fayeri* enterotoxin revealed the partial amino acid sequence that was used in this study. We propose that *S fayeri* toxin may be associated with weakness and muscle wasting in horses, and clinically affected horses may be identified by circulating *S fayeri* toxin antibodies that bind a partial peptide of *S fayeri* toxin. *Sarcocystis fayeri* sarcocysts may induce a pro-inflammatory response in horses. Detecting inflammation by serum CRP levels may be clinically useful because an elevated CRP was noted in diseased horses in this study, Table 2, section C and D. Clinically normal animals with antibodies against *S fayeri* toxin and an elevated CRP may indicate subclinical disease.

Although it remains unidentified, *S neurona* schizonts may produce sarcocystine. It is also possible that toxins, not schizonts, are responsible for CNS inflammation, the hallmark of histologic lesions in horses with EPM. Generally, host cell degeneration induced by sarcocystosis in intermediate hosts is inflammatory, this could be true in *S neurona* infections as well.[26] The toxin-associated pathology in neural tissues is one possible explanation for the lack of protozoa found in CNS tissues of animals with Sarcocystis encephalitis, including horses with EPM. It is possible that *S fayeri* enters the CNS. However, these parasites would have been detected by histopathology in diseased horses. We found no genetic similarity between the sequence of *S fayeri* toxin and *S neurona* protein sequences by a database search of NCBI and cannot speculate as to species specificity of sarcocystine.

This study supports the insufficiency of genus-detecting serological tests for clinical diagnosis of EPM due to Sarcocystosis in horses.[1] Sarcocystis induce encephalitis (without in situ parasites) in intermediate hosts and, therefore, the use of CSF fluid with genus-detecting tests in the diagnosis of EPM may be misleading. Antibodies against SFt were detected in CSF in one ataxic horse (data not shown). Because sera from horses with and without neuromuscular disease were seropositive for SFt and *S neurona* in this study it is possible that some infections are subclinical. Subclinical infections in horses may be detected by serotype specific antibodies associated with elevated CRP concentrations. This study associates sarcocystosis with inflammation, and it is possible that inflammation is the cause of clinical signs. The serodiagnosis of EPM is difficult because horses are often infected with both species of Sarcocystis, however in this study disease was noted in horses with single infections due to S fayeri.

DECLARATION OF ETHICS

The authors have adhered to the Principles of Veterinary Medical Ethics of the AVMA.

CONFLICTS OF INTEREST

Austin Li has no conflict of interest. Dr. Ellison is associated with Pathogenes and provides a consulting service for veterinarians with cases involving neurologic horses.

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