Development and Validation of an ELISA Detecting Sarcocystis fayeri Antitoxin in Horses with Equine Muscular Sarcocystosis (EMS)

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ABSTRACT

Disease due to *Sarcocystis fayeri* is associated with muscular sarcocystosis in horses and toxin-induced food poisoning in people. Sarcocysts of *S. fayeri* are found in muscles of horses on postmortem exam and generally considered an incidental finding. We report the presence of circulating *S. fayeri* antitoxin in a group of 32 clinically normal horses with an incidental finding of muscular sarcocysts on post-mortem examination. The histology examination was limited to three slides per horse in this study. *Sarcocystis fayeri* anti-toxin was present in horses with and without sarcocysts. The results of this report demonstrate similar sensitivity for detecting *S. fayeri* sarcocysts in muscle tissue by histopathology and by an *S. fayeri* anti-toxin ELISA (78% and 74%, respectively). These results indicate that detecting *S. fayeri* anti-toxin in equine serum has potential to be a useful pre-mortem screening test to detect the presence of muscular sarcocystosis in horses.

INTRODUCTION

Two distinct species of *Sarcocystis* produce sarcocysts in the muscles of horses. Thin-walled *S. bertrami* and thick-walled *S. fayeri* sarcocysts often are incidental findings in horses. *Sarcocystis fayeri* sarcocysts found in skeletal muscle were reported in association with neuromuscular disease.[1] Equine muscular sarcocystosis (EMS) is distinct from equine protozoal myeloencephalitis (EPM) because *S. neurona*, etiologic agent in EPM, fails to produce muscular cysts. Although not definitive, bradyzoites contained in *S. fayeri* sarcocysts are more distinct and less packed when compared to bradyzoites of *S. neurona* observed under light microscopy.

Sarcocysts are reported in skeletal muscles, esophagus, diaphragm, heart, and tongue of horses that ingest *Sarcocystis*

Table 1. *The presence of sarcocysts (SC) and horses seropositive for circulating S. fayeri anti-toxin (SFt) by treatment group are shown.*

Group	levamisole HCl	SC	SFt	SC +/ SFt -	SC -/ SFt +
1	0 mg/kg	5	4	2	1
2	1 mg/kg	5	7	0	2
3	3 mg/kg	7	6	2	1
4	5 mg/kg	4	3	3	2

fayeri-infected dog feces.[2] Two generations of pre-cyst stages (schizonts) occur between 10 and 25 days after infection. Immature sarcocysts are seen 55 days after infection and are infective to dogs by 77 days.[2] Cyst-producing equine *Sarcocystis* species are believed to be mildly pathogenic, although the production of clinical signs accompanying infections may vary among strains, duration of infection, co-infections, or the extent of infection.[2] *Sarcocystis* toxins are associated with the asexual stages of *Sarcocystis* found in intermediate hosts and recently *S. fayeri* anti-toxin was associated with neuromuscular disease in some naturally infected horses and enterotoxicity in people.[3,4,5]

Signs of acute *S. fayeri* sarcocystosis can include fever, lethargy, stiffness, and exercise intolerance. Animals infected with non-pathogenic strains may be asymptomatic. Signs of EMS can include apathy, muscle weakness and atrophy, neurological deficits, or dysphagia. A painful or stiff gait with a reluctance to move may also be present. Elevated creatinine kinase levels are reported in experimental infections.[2] Parasite-associated toxins are available to the horse's immune system after ingestion of sporocysts (during schizogony), and anti-toxin may develop prior to the development of sarcocysts.

Histological detection of *S. fayeri* may require special staining by stage-specific reagents or examining multiple slides per organ.[5] The purpose of this study was to determine the sensitivity of the *S. fayeri* ELISA (Sft-ELISA) when compared to histopathologically confirmed cases of EMS and determine the presence of anti-toxin to sarcocysts in healthy horses. Our hypothesis was the presence of *Sarcocystis fayeri* toxin in the serum of horses is a useful pre-mortem predictor of EMS.

MATERIALS AND METHODS

Post mortem examinations were conducted on 32 clinically normal horses involved in a drug safety study. One hematoxylin eosin stained section from the tongue, esophagus, and skeletal muscle from each horse was examined for a routine evaluation of drug toxicity. Serum was evaluated for anti-*S. fayeri* toxin (SFt) by ELISA after acclimation and entry into the study, Day 13 (T1), and Day 42 (T2), as previously described.3 Briefly, synthesized *S. fayeri* toxin (NCBI accession BAU71337.1) was coated on 96 well microtiter plates, blocked, and used to capture antitoxin from serially diluted sera. Control positive sera was obtained from histopathologically confirmed cases of EMS, negative sera were pooled from pre-suckle serum from three foals. Conjugated anti-horse secondary antibody was used to detect binding of the analyte. The sensitivity, specificity, and accuracy of the *S. fayeri* antitoxin ELISA test was evaluated using MedCalc Software (Acacialaan, Belgium) at 95% CI.

RESULTS

Sarcocysts were an incidental histopathological finding in the tongue, esophagus, and skeletal muscle in 21 horses on postmortem exam, Table 1. Circulating SF*t* was detected in 20 horses Table 1. Fourteen horses with sarcocysts were seropositive for SF*t*. Six horses that were SFt seropositive were histologically negative for sarcocysts and seven horses that were seronegative for SF*t* had histologically confirmed sarcocysts.

Creatine kinase levels were elevated in some horses, but were unrelated to sarcocystosis (data not shown). The status of the

Table 2. *Diagnostic test evaluation of muscle histopathology (SC) and S. fayeri anti-toxin (SFt) ELISA for the detection of EMS in 32 horses.*

95% CI	SC	SFt
True positive	21	20
True negative	5	5
sensitivity	77%	74%
specificity	100%	100%
prevalence	84%	84%
Positive predictive value	100%	100%
Negative predictive value	45%	42%
Accuracy	81%	78%

antibody levels against SFt did not change from Day 13 to Day 42 in 31 of 32 horses indicating exposure was prior to arriving at the test facility which was at least 14 days prior to Day 13.

Diagnosis of EMS by histopathology and detection of *S. fayeri* anti-toxin by ELISA were used to evaluate the diagnostic power of the tests at 95% CI and is shown in Table 2. Five true negative horses were horses that were negative on all tests. The sensitivity of histopathologic diagnosis using three muscle sections from each horse (77%) to determine status was superior to the sensitivity of SFt ELISA (74%) in these post-mortem samples from clinically normal horses, Table 2. The least sensitive diagnostic method was a diagnosis based on histopathology of a single skeletal muscle tissue section (26%), Table 3. The tongue and esophageal muscle sections were more reliable than skeletal muscle to identify EMS in these horses. Examining three sections was more reliable to detect sarcocysts, Table 3.

DISCUSSION

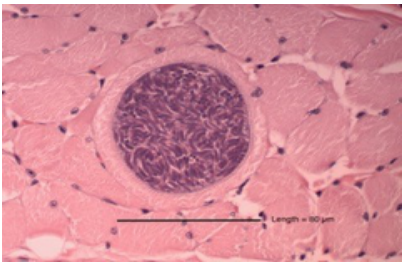
The sensitivity of post-mortem histopathology (77%) was slightly better than the pre-mortem SFt-ELISA (74%) for detecting EMS in horses. The advantage of SFt-ELISA is that the test is conducted in a live animal. The population of horses used in this study had a high prevalence of EMS, although this is not unusual. Reports of equine sarcocysts range from 4 to 93%. Higher infections are reported outside the USA, with 17.5% prevalence reported in the US (detected by histology, trypsin digestion, and bioassay).[1] In this study, control horses with EMS were asymptomatic. The cysts observed in muscle tissue were mature based on the absence of metrocytes that are present in young cysts (personal communication David Lindsay). The first exposure of these study horses to *Sarcocystis* was most likely a minimum of 77 days before post-mortem exam (Day 42, T2) based on descriptions of endogenous development (Figure 1) of *S. fayeri*, the most likely identity of the cysts, although molecular identification was not done.[2]

The time of exposure of these horses to *S. fayeri* was unknown, and exposure at the study facility was not ruled out but considered unlikely because immature cysts

Table 3. *A comparison of the diagnosis of sarcocysts by histopathology obtained from one section from each horse by location, tongue, esophagus, and skeletal muscle.*

95% CI	Histopath Tongue	Histopath Esophagus	Histopath Muscle
True positive	16	15	7
True negative	11	12	20
sensitivity	59	56	26
specificity	100	100	100
prevalence	84	84	84
Positive predictive value	100	100	100
Negative predictive value	31	29	20
Accuracy	66	63	38

Figure 1. *Sarcocystis fayeri sarcocyst in a muscle from a horse. Cross section of esophagus, hematoxylin and eosin stain; 400x.*



were not observed in the tissues. Our results support the observations of others that some strains of cyst-producing *Sarcocystis* are non-pathogenic to horses. It is possible that the study horses were infected with non-pathogenic strains because sarcocysts and *S. fayeri* anti-toxin were present in these clinically normal horses. There is known variability between *S. fayeri* strains and that can account for a difference in pathogenicity.6

Fourteen horses with sarcocysts had circulating anti-toxin, indicating exposure was at least 77 days prior to postmortem exam. Because the horses were obtained from various sources prior to entry into the study, the pathogenicity of the infecting strains and time of exposure is expected to differ in each horse. Exposure to *S. fayeri* not more than 3 weeks prior to arriving at the study facility could explain why six study horses were SFt seropositive and sarcocyst negative by histopathology. Alternatively, it is possible that the sarcocysts were not observed in the examined sections due to the small number of slides examined. If undetected sarcocysts were present, the results would indicate SF*t* could be associated with mature cysts and not just the schizont stages of infection.

Anti- SF*t* was detected in some, but not all, of the horses with EMS. It is possible that some of the horses were SF*t* seronegative and histopathologically positive because the strains infecting these horses were not releasing SF*t* or immune-reactive SF*t* was insufficient to produce circulating antibody. Toxins have been associated with *Sarcocystis* infecting humans, cattle, sheep, and other animals.[7,8,9,10] The *S. fayeri* toxin used in the ELISA was recovered from a horse with EMS was characterized and identified as an actin depolymerizing factor (ADF). The amino acid sequence for the toxin has high homology to ADF that was identified in other pathogenic protozoa including Toxoplasma gondii.[5] Actin depolymerizing factor is involved in modification of the cytoskeleton and motility of Apicomplexan parasites during infection.[5] These horses were not screened for *S. neurona* antibodies. It is unlikely that *S. neurona* would be detected by histopathology because *S. neurona* cysts have not been reported in horses and special stains were not used.

The effects of SF*t* are dose related in rabbits and a dose-related effect may be similar in naturally infected horses.[4,5] It was shown that the number of infected muscles and number of sarcocysts per muscle were significantly higher in horses with neuromuscular disease than control horses.[1] The number of muscle cysts may be related to strain pathogenicity, dose, or duration of infection. Although the prevalence of EMD in the study horses was high, the overall extent of infection was undetermined because only three sections were examined in each animal. If the sarcocyst load was low the horses may have had sufficient dose of SFt to stimulate immunity, but not disease.

It is interesting that *T. gondii* ADF-immunized mice are protected against *T. gondii* challenge.[5] Perhaps *S. fayeri* anti-toxin is protective against *Sarcocystis* challenge in horses. It was shown that co-infections with *Sarcocystis* sp. in horses were more common than single species infections in both normal horses and horses with neuromuscular disease.[3] Polyparasitism with *T. gondii* and *S. neurona* is an important factor contributing to disease in marine mammals indicating anti-ADF protection may be genus specific.[11]

It is important to distinguish horses with EMS with neuromuscular disease from horses with equine protozoal myeloencephalitis (EPM) because treatments for each con-

dition would be different, highlighting the need for species specific testing of samples from horses with suspected sarcocystosis. Currently there are no licensed treatments for EMS. *Sarcocystis* 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. The parasitemia is rapidly cleared and schizonts spread hematogenously to the viscera. Schizonts are found in the intestine, liver, lung, and kidney.

The terminally committed *S. fayeri* schizont enters muscles where it encysts. It is not believed that *S. neurona* infection in horses, the etiologic agent of EPM, terminates in a sarcocyst. And an ADF-like protein hasn't been described for *S. neurona*. It would be interesting to screen tissues from horses with EPM for bradyzoites using anti-ADF to detect cysts although it is still possible that ADF is expressed during schizogony and that would mean infected horses would be test-positive prior to cyst development.

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