KEY WORDS: Sarcocystis neurona, S. falcatula, horses, decoquinate

ABSTRACT

Sarcocystis neurona are apicomplexan parasites that can cause mortality in domestic and wild animals. Twelve antigen types are described for S. neurona, however just two phenotypes (SnSAG 1 and SnSAG 5) represent the majority of those that are associated with disease. A novel S. falcatula gene, antigenically indistinct from SnSAG 6, was cloned into an expression vector and the recombinant protein was used to determine the presence of antibodies SAG 6 phenotype infections in equine sarcocystosis. Additionally, antibodies to disease-associated SAG phenotypes of Sarcocystis neurona were assayed by indirect ELISA using sera from horses with a presumptive diagnosis of sarcocystosis. The lateral flow Sarcocystis neurona multiplex antibody detection kit (Immy, Norman, OK) was evaluated for the rapid identification of Sarcocystis antibodies. One hundred forty-one horses (141) with a presumptive diagnosis of equine protozoal myeloencephalitis were treated with decoquinate (0.5mg/kg) and levamisole (1 mg/kg) in an oral paste for 10 days and monitored for a treatment response. Successful treatment of equine sarcocystosis was determined by a reduction in clinical signs by clinical neurological exam (93.6%) and a reduction in antibodies 4-6 weeks post treatment (89.3%). The detection of antibody against SAG’s 1, 5, 6 and a response to decoquinate/levamisole identified horses with clinical EPM. The rapid detection of SAG phenotype facilitated treatment and evaluation of clinical response.

INTRODUCTION

Sarcocystis neurona are pathogenic protozoa that can cause mortality in domestic and wild animals. Three unique phenotypes of S. neurona have been described and two of these (SnSAG 1 and 5) predominate in animal disease. In one study twelve antigen types portrayed a simple population genetic structure for S. neurona, and a few dominating clonal lines in North America were of veterinary importance. After ingestion, S. neurona oocysts excyst in the gut tissues. Merogony produces asexual merozoites that rapidly travel to the viscera via a parasitemia. Horses are considered aberrant hosts for S. neurona because the asexual cysts are not found in equine muscles. The presence of merozoites in neural tissues and/or inflammation that results in recognized pathology are considered the source of signs associated with equine protozoal myelo-
encephalitis (EPM). Immune-mediated pathology rather than parasite mediated pathology was considered as one source of signs observed in oocyst challenge experiments because parasites were not demonstrated in these studies. The culture of organisms from neural tissues remains the gold standard used to verify clinical EPM. However, it is generally accepted that the transport model used by Ohio State induced clinical EPM in the absence of evidence of merozoites. Confirmed field cases of EPM were identified by recovering S. neurona merozoites from the central nervous system of clinically ill horses by in vitro culture, the first isolation was made by J. P. Dubey. Experimental clinical EPM was induced with a merozoite challenge model and this challenge model resulted in parasites recovered from the CNS and the infections verified by in vitro culture. The current dogma that merozoites elicit permanent neural damage may be erroneous. Parasites can enter the CNS as shown in field and experimental (merozoite) cases—however complicated inflammatory mechanisms induced by protozoal infections may contribute significantly to clinical signs.

The opossum (Didelphis virginiana) is the source of infectious oocysts for horses. Highly similar DNA sequences for 18s rRNA genes amplified from opossum sporocysts and S. neurona merozoites led Dr. Claire Fenger and her associates at the University of Kentucky to suggest that the opossum was likely the definitive host of S. neurona. This prompted researchers from the University of Florida (Dame and MacKay) to suggest that S. neurona and S. falcatula were synonymous. The idea was based on greater than 99% homogeneity between a 742-bp segment of the 18S rRNA gene from S. falcatula and S. neurona (SnSAG1) amplified by PCR from oocysts that were collected from opossums. Additional studies that failed to induce clinical EPM in horses that were challenged with an authenticated population of S. falcatula sporocysts led these researchers to suggest that the organisms were not synonymous and, further, bioassay was a significant parameter discerning species differences. The inability of S. falcatula to infect horses in their studies was based S. neurona immunoblots that failed to detect antibodies in serum and CSF post-challenge and the absence of clinical signs of EPM. Confirmed infections of horses by using sporocysts have been elusive and the UF studies were based on oocyst challenge. Further work done by Heskett and MacKay concluded that sporocyst infection challenge may not reliably result in CNS infection.

Serum samples obtained from a Missouri horse with confirmed clinical EPM (SnSAG 5 phenotype) bound antigens of S. falcatula when S. falcatula blotted antigens were probed with the serum. These data also indicated that there are differences in immunoblot patterns between two S. falcatula strains when this serum and specific rabbit sera were used. The ability to detect S. falcatula antibodies in serum/CSF may depend not only on the species of Sarcocystis but also the strains used in the antigen preparation. Possibly antigen selection is responsible for the failure to detect seroconversion in the University of Florida challenge studies.

An advance in genotyping has allowed better analysis of relatedness between S. neurona strains. Based on limited available genetic data, sequence-level analysis of five surface antigens (Ag) genes (SnSAG 1, 3, 4, 5, and 6) and nine microsatellite (MS) markers identified 12 Ag types and 33 MS types among S. neurona-infected samples based on the allele combinations detected at each locus. The resolution of strains of S. neurona is achieved using genetic sequence analysis. The distinction of phenotype is achieved by detecting specific antibodies against recombinant proteins SnSAG 1, 5, and 6 because these surface antigens are mutually exclusive genes of S. neurona. Sarcocystis neurona SnSAG 1 and 5 primers are S. neurona specific while SnSAG 2-3-4-6 are amplified by S. neurona and S. falcatula DNA. Due to the highly conserved
orthologs to SAG 2, 3, 4 in the closely related *S. neurona* and *S. falcata* antibody detection of these phenotypes would not be useful in diagnostic tests. \textsuperscript{xvi}

One goal of this study is to clarify the ability of horses to develop antibodies against *S. falcata*—as demonstrated in the Missouri isolate, but not demonstrated in the UF *S. falcata* challenge studies. Two *Sarcocystis falcata* isolates were subjected to genetic analysis using the SAG primers described by Wendte.\textsuperscript{xvi} Lung tissues from birds that were infected with opossum oocysts described as *S. falcata* that were obtained from the Florida horse infection challenge were placed in vitro culture.\textsuperscript{*} An additional strain of *S. falcata* (Cornell) was characterized. DNA samples from the cultured merozoites were analyzed by high resolution genetic typing. We report the results of the genetic sequence analysis that identified a novel gene, SfSAG 6, from the Florida isolate but not present in the Cornell isolate. The gene was named SfSAG 6 due to the high similarity to SnSAG 6 of *S. neurona*. The recombinant SfSAG 6 protein was assayed by ELISA and lateral flow tests to determine the presence of antibody in equine serum from horses with clinical EPM.

Horses with a diagnosis of EPM that undergo treatment are often unresponsive to triazine or pyrimethamine/sulfadiazine drugs. Often treatment of EPM involves empirically increasing the dose and duration of these drugs beyond approved label recommendations due to a lack of treatment options. The disease EPM involves immune mechanisms that include inflammation. Triazine based treatment protocols do not address the inflammatory component of EPM. Failure to address the inflammatory component of disease associated with EPM may be a source of treatment failure observed in clinical cases. Levamisole-HCL is a synthetic imidazothiazole with a long history of immune modulating properties in humans and animals.\textsuperscript{xvii} Levamisole was used in horses with chronic obstructive pulmonary disease (heaves) and onchocerciasis but the ability to change a Th2 to a Th1 response may benefit horses with EPM.\textsuperscript{xviii} Additionally, Sajid suggested that levamisole-HCL restores immune function rather than stimulating a response to above normal levels. Due to these immune modulating effects in other species levamisole was considered a possible adjunct to an effective EPM treatment and was added to decoquinate therapy for this study. Decoquinate may afford a treatment option for *Sarcocystis neurona* in horses based on in vitro data in a patent submitted in 2001. \textsuperscript{xix} This publication reported that SnSAG 1 and SnSAG 5 phenotypes required ten times more triazine drugs than decoquinate to kill the protozoa when tested in vitro. Additionally a reduction in these merozoites, but not the elimination of the organisms, in the flask was reported. The difference in phenotype between strains was unrecognized in 2001. The highly efficient elimination of both phenotypes by decoquinate in Lindsay’s experiments showed promise for the effective treatment of EPM in horses.

**MATERIALS AND METHODS**

**Equine Sera**

One hundred sera were obtained from a normal population of horses and the sera were tested for the presence of antibodies to *S. neurona* SnSAG 1, 5, and SfSAG 6. One hundred serum samples that were submitted for ELISA testing obtained from horses that had a presumptive diagnosis of EPM based on clinical exam were examined for the distribution of antibodies against SnSAG 1, 5, and 6. Horses with a presumptive diagnosis of EPM based on clinical signs of ataxia determined by a veterinarian and had a titer of >16 determined by ELISA were entered into a field trial. Serum was obtained at least twice from these clinically ill horses, pre-treatment and 4-6 weeks following treatment.

Monospecific polyclonal antibodies were produced against each recombinant protein, SnSAG 1 and 5 and SfSAG 6. Three horses were tested and found to have no serum antibodies against SAG 1, 5, or 6. One horse
was vaccinated with rSnSAG 1, a second horse was vaccinated with rSnSAG 5, and a third horse was vaccinated with rSfSAG 6. Antibodies against recombinant proteins were produced by vaccination using 50µG of the recombinant protein in Polygen (MVP Labs, Ia.) adjuvant give by IM injection to a horse. The vaccination was given to each seronegative horse (determined by ELISA) three times, three weeks apart. Serum was obtained by jugular venipuncture three weeks following the last vaccination. These sera were used as control positive samples in the ELISA assay. The pre-vaccination sera were used as negative controls in the ELISA assay.

DNA and recombinant proteins
The DNA samples and genetic sequence for SnSAG 1, 5, and 6 were previously described. *Sarcocystis neurona* SnSAG 1 gene sequence was described in a work done for the authors PhD thesis. The SnSAG 5 sequence was obtained from results reported from the strain isolated from the Missouri horse. The SnSAG 6 gene was described by Wendte. *S. falcatula* DNA was obtained from merozoites from bird lung placed into in vitro culture by the author from studies conducted by Dr. Tim Culter for a masters thesis and used in the University of Florida challenge experiments. Identification of SfSAG 6 by PCR was done as previously described in (Wendte) by Dr. Jared Wendte at National Institutes of Health. The Sf-SAG 6 sequence is shown in Figure 1. The cloning and expression of recombinant SnSAG1, 5, 6 was commercially obtained (GenScript Piscataway, NJ).

The recombinant proteins were verified as specific and non-cross reactive in immunoblots as well as ELISA tests using monospecific polyclonal antibodies made in horses as control sera. The control sera also bound specific recombinant proteins in the lateral flow *Sarcocystis neurona* multiplex antibody detection kit (Immy, Norman Ok.).

Serum testing
Recombinant antigens SnSAG 1, 5, and SfSAG 6 were diluted in carbonate/bicarbonate buffer (Sigma Aldrich) to obtain a 1µG/mL solution. One hundred µL was absorbed to each well of a 96 well microtiter plate overnight at 4C. The plates were blocked with 1% bovine serum albumin (Sigma-Aldrich St. Louis, Mo.) overnight at 4C. Each sera were serially diluted by two fold dilutions and incubated for one hour at room temperature. The plates were washed and a standard ELISA protocol followed as previously reported xxx. The reciprocal of the last dilution showing a positive reaction was recorded as the titer.

In a clinical field trial horses with a presumptive diagnosis of EPM based on clinical signs detected by a veterinarian were tested for the presence of serum antibodies against SnSAG 1, 5, and SfSAG 6 by
diagnosis of EPM, with a positive reaction at 1:16 or higher, were treated with 0.5mG/kg decoquinate and 1 mG/kg levamisole PO for 10 days. A second clinical exam was performed by the same veterinarian to document a change in clinical signs post treatment. A second antibody titer was obtained 4-6 weeks after treatment and a post treatment serum titer was determined.

Some serum was also screened using a lateral flow Sarcocystis neurona multiplex antibody detection kit as described in the manufacturer instructions (Immy).

RESULTS

Presence of antibodies in equine serum

Equine serum was tested for the presence and distribution of antibodies against S. neurona by phenotype ELISA. Fifty-nine percent of the sera contained antibodies at a dilution of 1:16 or higher. The predominant infections detected in normal horse sera were SfSAG 6 (26%). Mixed infections were present (18%) in the sample set. Sarcocystis neurona SnSAG 5 antibodies were found in 11% of the serum samples and 4% contained SnSAG 1 antibodies. Sera tested for the presence and distribution of antibodies against S. neurona to confirm a diagnosis of EPM (clinical exam by a veterinarian) were positive in 84% of the samples. Mixed infections predominated (48%) while Sn-(22%). Antibodies against SfSAG 6 were detected in 8% of the samples and SnSAG 5 antibodies were detected in 6% of the samples. Serum antibodies against SnSAG 1, 5, or SfSAG 6 were detected by S. neurona multiplex antibody detection kit when antibodies were present at a dilution of 1:20 or greater. The strips were positive at a 1:20 dilution, and weak reactions were seen when antibodies were detected by ELISA at 1:8-1:16. There was no evidence of antibody on strips for sera that tested at 1:8 or less by ELISA.

Field trial: Clinical response to treatment based on clinical exam

Data was obtained from 246 horses based on records for sera submitted for SAG 1, 5, 6 ELISA testing. Horses that received decoquinate/levamisole were documented by examining records for filled prescriptions for the treatment. Decoquinate/levamisole was administered to 195 horses at 0.5 mG/kg decoquinate/ 1mG/kg levamisole in a flavored base for ten days. No adverse events were reported associated with the treatment for 195 horses. Serum from horses obtained pre-treatment and 4-6 weeks post-treatment was available from 146 horses. Clinical evaluation forms were completed by the attending veterinarian before and after treatment and accompanied the serum submis-

<table>
<thead>
<tr>
<th>Table 1. Tabulated results for 100 sera from clinically normal horses and 100 horses with a presumptive diagnosis of EPM.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seronegative</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>EPM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. Tabulated results for 141 horses receiving decoquinate 0.05mG/kg and levamisole 1mg/kg for ten days.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ELISA. The horses with a presumptive</strong></td>
</tr>
<tr>
<td><strong>Decreased clinical signs</strong></td>
</tr>
<tr>
<td>Decreased clinical signs</td>
</tr>
<tr>
<td>132/141</td>
</tr>
<tr>
<td>93.6%</td>
</tr>
</tbody>
</table>

|  | **SnSAG1** | **SnSAG5** | **SnSAG6** |
| Only SAG 1 infection | 132/141 | 126/141 | 19/141 |
| Only SAG 5 infection | 8/141 | 5/141 |
| Only SAG 6 infection | 5/141 |

|  | **SnSAG1** | **SnSAG5** | **SnSAG6** |
| Only SAG 1 infection | 93.6% | 89.3% | 13.4% |
| Only SAG 5 infection | 5.6% | 3.5% |
| Only SAG 6 infection | 3.5% |
sion for 141 horses that received decoquinate/levamisole for clinical signs of EPM. Missing information on the submission form was obtained by a phone call to the veterinarian that submitted the serum for analysis.

A positive clinical response was reported by the attending veterinarians in 132 of the 141 decoquinate/levamisole treated horses. A drop in titer was reported in 126 of the post-treatment sera (when compared to the pre-treatment titer) obtained from paired serum samples that were run before treatment and again 4-6 weeks post-treatment. The *Sarcocystis neurona* multiplex antibody detection kit was with 50 serum samples using the manufacturer recommendations of 1:20 dilution in the supplied buffer. The strips were positive at a 1:20 dilution, and weak reactions were seen when antibodies were detected by ELISA at 1:8-1:16. There was no evidence of antibody on strips for sera that tested at 1:8 or less by ELISA.

**DISCUSSION**

Determining the presence of serum antibodies against *S. neurona* and distinguishing the phenotype that caused the infections can assist the veterinarian in treatment decisions for the horse with EPM. We show that sera from some normal horses and those with clinical signs EPM contain antibodies detected by SAG 1, 5, 6 ELISA. Strain resistance to triazine drugs has been reported and phenotyping before and after treatment with antibodies were detected by ELISA at 1:8-1:16. There was no evidence of antibody on strips for sera that tested at 1:8 or less by ELISA.

We detected antibodies against Sarcocystis SAG 6 organisms; however it is not possible to distinguish SnSAG 6 and SfSAG 6 organisms with antibody tests, the sequence for both genes are highly similar and cross reactions are anticipated. It is unclear the role *S. falcatula* plays in clinical EPM. Prior experimental evidence showed that authenticated *S. falcatula* oocysts derived from opossum feces didn’t infect horses and antibodies against *S. neurona* were not produced. It is not surprising that challenge studies failed to show antibodies induced in *S. falcatula* studies because the sera was screened against antigens of *S. neurona*. Alternatively, the oocyst model didn’t induce sarcocystosis that resulted in antibody production. Clinical EPM with isolation of the organism from the CNS was not reproducible in other oocyst models despite evidence that merozoites infect visceral organs of the horse. Heskett and MacKay concluded that challenging horses via oocysts may not reliably result in CNS infections. Merozoites enter the CNS of horses and produce clinical signs of EPM in the Trojan horse--merozoite model. The methods of induction of disease or strain differences are possible factors in differences between oocyst and merozoite induced infection studies.

It is possible that *S. neurona* SAG 6 can infect horses while *S. falcatula* cannot. In this study phenotypes of *S. neurona* that infect horses were determined by specific ELISA tests using recombinant proteins. Antibodies against SnSAG 1, 5, and 6 were detected in normal horses indicating that infections that resolve are common. It is interesting that only 4% of normal horses have antibodies against SnSAG 1 strains which dominate the isolations published from field infections. We detected the presence of *S. falcatula* SAG 6 antibodies in normal and diseased horses. This was not surprising because *S. falcatula* specific antibodies were detected by Marsh in a report that identified *S. neurona* SAG 5 phenotype merozoites from the CNS tissues of a Missouri horse. Studies by Wendte indicate the majority of disease in animals is due to SnSAG 1 or SnSAG 5 phenotypes, and only these phenotypes have been recovered from the CNS of diseased horses.

In this study the distribution of *S. neurona* antibodies in normal horses is 59% while the distribution in horses with a presumptive diagnosis of EPM is 84%. Sarcocystis SAG 6 serum antibodies were detected in 3.5% of clinically ill horses undergoing treatment for EPM indicating that *S. neurona* SAG 6 phenotypes can cause disease. The results of SAG 6 testing can be due to cross-reaction between SnSAG 6 and SfSAG 6 if S.
*Falcatula* causes disease in horses. Twenty-six percent of the sera from animals that did not have clinical signs of EPM did have SAG 6 antibodies. The difference between horses with and without disease may be due to species, *S. neurona* eliciting disease while *S. falcatula* does not (as was previously reported). However, *S. falcatula* antibodies were detected in at least one natural case of EPM—the Missouri horse, and SAG 6 antibodies are detected in horses when SfSAG 6 recombinant proteins are used for detection.

The use of bioassay in budgies or immune deficient mice, or genetic sequence analysis, is required to distinguish *S. neurona* from *S. falcatula* and it’s possible that horses can discern the difference between these organisms. Both *S. neurona* and *S. falcatula* are identical in antibody tests using antigens SAG 2 and 3, (SAG 4 is variable in some *S. falcatula* strains, data not shown) therefore these diagnostic antigens are not specific for *S. neurona*. Horses with antibody to SAG 6 and clinical signs of disease responded to treatment indicating that SAG 6 strains of *S. neurona* are important in clinical EPM. However, other sarcocystis that do not induce disease would be detected by tests that are not specific for *S. neurona* and phenotype profiling would be important to discern the difference.

In order to determine if signs of EPM in clinically ill horses were related to presence of infection we treated horses with an effective anti-protozoal agent, decoquinate, and monitored the antibody response in serum by phenotype ELISA. Decoquinate was chosen for this study because toltrazuril and diclazuril have shown resistance in *S. neurona* infections both in vitro and in vivo. In this study 141 horses were treated with diclazuril/levamisole for 10 days and 132 horses showed a positive response to treatment with alleviation of clinical signs and a reduction in antibody titer that was recorded for 126 horses.

Levamisole was indicated in the treatment protocol because *S. neurona* infections in horses (both natural and induced infections) cause nervous system histopathological lesions that might be attributed to immune mechanisms and not the physical presence of parasites. We showed direct effects on the immune system in early infections using our induced model that were also present in natural infections.xx, xxi, xxii At moderate doses levamisole is an immune suppressant, but at low doses levamisole modulates a suppressed immune response (but not an appropriate immune response). The anti-helminthic levamisole was used in horses for atopic disease at 8 mg/kg before other drugs became available commercially. xxiii Mongan NP, Baylis HA, Adcock C, Smith GR, Sansom MS, Sattelle DB. An extensive and diverse gene family of nicotinic acetylcholine receptor alpha subunits in Caenorhabditis elegans. Receptors Channels. 1998;6(3):213–228

Treatment crisis, possibly due to allergic responses to protozoal antigens, are seen with anti/protozoal therapy in horses with clinical EPM and these crisis reactions were anticipated in this study. The inflammatory reaction to parasites is a complicated process that can elicit clinical signs. Some researchers believe that inflammatory histological lesions documented in the CNS of infected animals are due to the presence of parasites despite lack of detection (personal communication, Mike Grigg). It is also possible the lesions are due to the inflammatory cascade that does not require parasites to be present in the CNS. Parasites were eliminated by the anti/protozoal drug decoquinate as shown by a reduction in titer post treatment in this study. The marked improvement in clinical signs may be partially due to the effects of levamisole as an immune modulator or effects on other neurobiological mechanisms. One such effect is the possibility that *S. neurona* or the immune response to the organism damages some upper motor neuron function that is responsive to levamisole therapy. An area of ongoing research is examining the loss of levamisole receptors on the equine upper motor neuron in diseased
horses when compared to normal horses. Also understanding the genetic distribution of nicotine acetylcholine receptors (those which respond to levamisole) in the horse could indicate that susceptibility to EPM does have a genetic basis. The presence of these receptors does have a genetic basis in C. elegans.xxiii

Based on the results of this study a reasonable approach to the diagnosis of EPM in the horse is the detection of phenotype by serum ELISA and a response to treatment with decoquinate and levamisole for a 10 day period.

*a kind gift of Ellis Greiner, University of Florida

REFERENCES