Decoquinate Combined with Levamisole Reduce the Clinical Signs and Serum SAG 1, 5, 6 Antibodies in Horses with Suspected Equine Protozoal Myeloencephalitis

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ABSTRACT

Sarcocystis neurona is an apicomplexan parasite that can cause mortality in domestic and wild animals. It is an important cause of the neurological disease equine protozoal myeloencephalitis (EPM) in the Americas. Different surface antigen (SAG) phenotypes have been observed for *S. neurona*, and not all isolates of *S. neurona* contain the genes (SnSAG) to encode all SAGs. Recent studies indicate the presence of antibodies to the antigens SAG 1 and SAG 5 are present in most cases of clinical disease. Using sera from horses with a presumptive diagnosis of EPM, we examined 141 horses for antibodies to disease-associated SAG phenotypes of *S. neurona* using an indirect ELISA employing recombinant SAG's 1, 5, and 6 as antigens. An immunochromatic *Sarcocystis neurona* multiplex antibody detection kit (Centaur, Olathe, KS.) was evaluated for the rapid detection of antibodies to SAG 1, 5, and 6 of *S. neurona*. One hundred forty-one horses with a presumptive diagnosis of EPM were treated with a combination of decoquinate (0.5 mg/kg) and levamisole (1 mg/kg) in an oral paste, Oroquin-10 (Francks Compounding Labs, Ocala, Fl.), for 10 days and monitored for a treatment response. Successful treatment of EPM was determined by a reduction in clinical signs by clinical neurological examination and a reduction in antibodies 4-6 weeks post treatment. A reduction in clinical signs was seen in 132 (93.6%) horses and reduced antibody titers were observed in 126 (89.3%) horses. The detection of antibody against recombinant SAG's 1, 5, 6, and a response to decoquinate/ levamisole identified horses with clinical EPM. The rapid detection of SAG phenotype using the multiplex antibody detection kit facilitated identification of significant antibodies and evaluation of a clinical response.

INTRODUCTION

Sarcocystis neurona is a pathogenic protozoan that can cause mortality in domestic and wild animals, and is transmitted by opossums (*Didelphis virginiana*). It is responsible for causing equine protozoal myeloencephalitis (EPM) in horses in the Americas. Three unique phenotypes of *S. neurona*

have been described and two of these genes (SnSAG 1 and SnSAG 5) predominate in isolates from EPM cases.[1] After ingestion, *S. neurona* oocysts excyst in the intestinal tissues. Asexual reproduction by merogony produces merozoites that rapidly travel to the viscera via a parasitemia.[2]

Horses are considered aberrant hosts for *S. neurona* because the asexual sarcocyst stages 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 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.[3] The culture of organisms from neural tissues remains the gold standard used to verify clinical EPM. Our central hypothesis is that specific SAG antigens are more common in horses with a presumptive diagnosis of EPM. In the present study, we observed phenotype by detection of serum antibodies using ELISA, and revealed a reduction of these antibodies using decoquinate-levamisole to treat EPM. Horses with a diagnosis of EPM that undergo treatment are frequently unresponsive to pyrimethamine/sulfadiazine or triazine 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 and pyrimethamine/sulfadiazine based treatment protocols do not address the inflammatory component of EPM. Failure to address the inflammatory component of disease associated with EPM may be one source of treatment failure observed in clinical cases. Levamisole-HCl has been used as an immune modulator in other species.[4] Levamisole was considered a possible adjunct to an effective EPM treatment, and was added to decoquinate therapy for this study. Levamisole-HCL is a synthetic imadizothiazole with a long history of immune modulating properties in humans and animals.[5] Levamisole was used in horses as an anthelmintic and an immune modulator.[6] Researchers have suggested that levamisole-HCL restores immune function rather than stimulating a response to above normal levels increasing the possible positive effects of this drug in cases of EPM.[5] Decoquinate may afford a treatment option for *S. neurona* in horses based on *in vitro* data in a patent submitted in 2001.[7] The highly efficient elimination of parasites by decoquinate in Lindsay's experiments showed promise for the effective treatment of EPM in horses due to the protozoa-cidal actions of decoquinate over the statically acting triazines.

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* surface antigens using recombinant SAG 1, 5, and 6 proteins. One hundred serum samples that were submitted for ELISA testing obtained from horses that had a presumptive diagnosis of EPM based on clinical examination 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 were 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 equine antibodies were produced against each recombinant protein, SnSAG 1 and 5, and SfSAG 6 to generate positive control sera. Three horses were tested and found to have no serum antibodies against SAG 1, 5, or 6. One horse was vaccinated with rSAG 1, a second horse was vaccinated with rSAG 5, and a third horse was vaccinated with rSAG 6. Antibodies against recombinant proteins were produced by vaccination using 50 μg

of the recombinant protein in Polygen (MVP Technologies, Omaha, NE.) 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 sequence was obtained from SnSAG 1 and SnSAG 5 as previously described.[8,9,10] The SnSAG 6 gene was described by Wendte.[11] The DNA for the production of SAG 6 in this study was obtained from the bioassay of oocysts in horses and birds.[12] The recombinant proteins (GenScript Piscataway, NJ) were verified as specific and non-cross reactive in *S. neurona* immunoblots as well as ELISA tests using monospecific polyclonal antibodies made in horses as control sera (data not shown).

Serum Testing

Recombinant antigens SAG 1, 5, and 6 were diluted in carbonate/bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) 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.[13] The reciprocal of the last dilution showing a positive reaction was recorded as the titer. The sensitivity and specificity of the SAG 1 ELISA in experimentally induced acute and chronic EPM has been reported.[14]

The commercially available immunochromatic *Sarcocystis neurona* multiplex antibody detection kit (Centaur Inc, Olathe, KS) was used to compare the presence of antibodies and phenotype results obtained in the ELISA. The strip tests were validated using control positive sera, sera from vaccinated horses, and 75 experimentally infected horses (data not shown). Sera from 50 horses from the field trial in this study were used to compare the results to the ELISA test results.

Clinical Trial of Decoquinate and Levamisole

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 recombinant SAG's 1, 5, and 6 by ELISA. The horses with a presumptive diagnosis of EPM, with a positive reaction at 1:16 or higher, were treated with 0.5 mg/kg decoquinate and 1 mg/kg levamisole PO (Oroquin-10) for 10 days. A second clinical examination was performed by the same veterinarian to document a change in clinical signs at the end of treatment. A second antibody titer was obtained 4-6 weeks after treatment and a post treatment serum titer was determined for each horse in the clinical trial.

RESULTS

Presence of Antibodies in Equine Serum

Equine sera from a population of normal horses were 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 SAG 6 (26%). Mixed infections were present (18%) in the sample set. *Sarcocystis neurona* SAG 5 antibodies were found in 11% of the serum samples and 4% contained SAG 1 antibodies. Sera tested for the presence and distribution of antibodies against *S. neurona* to confirm a diagnosis of EPM (clinical examination by a veterinarian) were positive in 84% of the samples. Mixed infections predominated (48%), while SAG 1 was the predominant single infection (22%).

Antibodies against SAG 6 were detected in 8% of the samples and SAG 5 antibodies were detected in 6% of the samples. Serum

Table 1. Tabulated results for 100 sera from clinically normal horses and 100 horses with a presumptive diagnosis of EPM using the ELISA test.

	Seronegative	Seropositive	Mixed Inf.	SAG1	SAG 5	SAG 6
Normal	41%	59%	18%	4%	11%	26%
Suspect EPM	16%	84%	48%	22%	6%	8%

Table 2. Tabulated results showing clinical and immunological reactions for 141 horses receiving decoquinate 0.05 mg/kg and levamisole 1 mg/kg for ten days.

Decreased clinical signs	Decrease in titer post treatment	Only SAG 1 infection	Only SAG 5 infection	Only SAG 6 infection
132/141	126/141	19/141	8/141	5/141
93.6%	89.3%	13.4%	5.6%	3.5%

antibodies against SAG 1, 5, or SAG 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.

In the Field trial, clinical response to treatment was 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 Oroquin-10. Oroquin-10 was administered to 195 horses so that 0.5 mg/kg decoquinate/ 1mg/kg levamisole in a flavored base for ten days was administered. 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 at the end of the 10-day treatment. The examination forms accompanied the serum submission for 141 horses that received Oroquin-10 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 Oroquin-10 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 used with 50 serum samples at 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. There was no difference between detection of antibodies for the ELISA at 1:20 or the strips at the 1:20 dilution.

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 of EPM contain antibodies that are detected by the SAG 1, 5, 6 ELISA. A rapid reduction in clinical signs noted within the 10 day treatment period that was accompanied by reduction in serum titers measured 4-6 weeks after treatment indicated a good treatment response in horses with clinical EPM confirming the source of the infections.

Table 3. *Tabulated results showing correlation between phenotype ELISA and S. neurona multiplex antibody detection strips.*

	ELISA at 1:20 dilution	Multiplex strip test result
Pre-treatment 50 horses	50/50 positive	50/50 positive
Post-treatment 50 horses	50/50 negative	50/50 negative

The properties of static drugs like the triazines could be a factor in the lack of treatment response. Relapse may occur if horses are not treated long enough with triazine drugs. Reactivation of the infection may occur during periods of unusual stress. The conventional chemotherapy regimens do not completely remove all disease-causing parasites from the central nervous system. Also, if too-low concentrations of triazine drugs are used, intracellular stages of the parasites are not killed.[7] Phenotyping by detection of SAG 1, 5, and 6 antibodies in serum before and after treatment in unresponsive cases can indicate treatment failure if a reduction in titer does not occur. The more rapidly acting cidal antiprotozoal drug decoquinate produced a reduction in clinical signs within the 10 day treatment period.

We detected antibodies against Sarcocystis SAG 6 organisms in both normal horses and those with a clinical diagnosis of EPM, verifying the importance of this phenotype in infections. However it is not possible to distinguish between SAG 6 phenotypes of *S. neurona* and *S. falcatula* with antibody tests; the sequence for both genes are highly similar and cross reactions are anticipated.[15] It is unclear what 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.[12] 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*.[12] 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.[2,16] Merozoites enter the CNS of horses and produce clinical signs of EPM in the Trojan horse--merozoite model.[17] The methods of induction of disease or strain differences are possible factors in differences between oocyst and merozoite induced infection studies.

In this study, phenotypes of *S. neurona* that infect horses were determined by specific ELISA tests using recombinant proteins. Antibodies against SAG 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 SAG 1 strains, which dominate the isolations published from field infections. However, in horses with a presumptive diagnosis of EPM, we noted that 22% had only SAG 1 reactive antibodies, which is supported by the predominance of this phenotype found in field isolations. 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.[18] Studies by Wendte indicate the majority of disease in animals is due to SAG 1 or SAG 5 phenotypes, and only these phenotypes have been recovered from the CNS of diseased horses.[1] Because the SAG 6 phenotype antibodies do not cross-react with SAG 1 and SAG 5 antigens and *S. falcatula* is not expected to cause clinical disease in the horse, we may be detecting equine disease associated with *S. neurona* SnSAG 6 containing organisms.

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 proved disappointing in treating clinical EPM in the field. In this study, 141 horses were treated with decoquinate-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.[19,20,21] It is generally accepted that the transport model can induce clinical EPM in the absence of evidence of merozoites in the CNS. 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.[22] 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.[17] The current belief that merozoites alone elicit direct neural damage causing clinical signs may be erroneous. Complicated inflammatory mechanisms induced by protozoal infections may contribute significantly to clinical signs. 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 is recognized for immune modulation in horses.[6]

Treatment crisis, is a transient worsening of clinical signs early in treatment (possibly due to allergic responses to protozoal antigens) and is 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. Most researchers believe that inflammatory histological lesions documented in the CNS of infected animals are due to the presence of parasites despite lack of detection. It is also possible the lesions are due to the inflammatory cascade that does not require parasites to be present in the CNS.

The anti-protozoal drug decoquinate was effective 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*.[5]

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.

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