Development of an ELISA to Detect Antibodies to rSAG1 in the Horse

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ABSTRACT

The definitive diagnosis of *Sarcocystis neurona* infection in the horse is challenging. The production of specific tools and techniques that aid in the identification of horses with parasitic encephalitis are needed for the diagnosis of EPM (equine protozoal myeloencephalitis) caused by *S. neurona*. A candidate antigen for the detection of *S. neurona* infections in horses is the immunodominant surface antigen, SnSAG1, of *S. neurona*. An enzyme linked immunosorbent assay (ELISA) was developed and used to measure antibodies to rSnSAG1 (recombinant SAG1). This report describes the use of SAG1 ELISA to detect novel serum and CSF antibodies to SnSAG1 in six experimentally infected horses that showed clinical signs of EPM after challenge. Horses (n = 330) with neurologic ataxia or lameness of undetermined cause, presumptive EPM, were tested, and antibodies were found in 85.4% of the horses. Random serum samples from a normal population were evaluated by SAG1 ELISA, and 6% were found to test positive at a titer greater than 200.

INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a serious neurologic disease of horses. The principal causative agent is *Sarcocystis neurona*.[1] Definitive antemortem diagnosis of EPM is challenging, because exposure to *S. neurona* does not always produce clinical disease.[2–4] Although there are no universally accepted methods for diagnosing EPM, a presumptive diagnosis is based on neurologic signs that are not attributable to another disease. Supportive evidence for a presumptive diagnosis of EPM is either the detection of *S. neurona* or antibodies to the parasite in the cerebrospinal fluid (CSF) collected from a horse with neurologic abnormalities. The methods that have been used to identify exposure to *S. neurona* include detection of antibodies by immunoblot, detection of DNA from *S. neurona* by PCR, or identification of the organisms in tissues using immunodiagnostic techniques or in vitro culture.[5–8] Sometimes diagnosis of disease is based on response to drugs that are believed to kill the parasite that causes EPM.

The most common test used to support the diagnosis of horses that have been infected with *S. neurona* is a commercial immunoblot assay. Immunoblot analysis of serum and CSF has been widely used to identify antibodies to *S. neurona*; the estimate is that from 30% to

60% of horses have been previously exposed to *S. neurona* based on immunoblot results.[4,5] Some researchers have suggested that a change from no detectable antibodies in CSF to *S. neurona* by immunoblot to a positive test is evidence of infection when accompanied by clinical signs of disease.[3] This hypothesis presumes that intrathecal antibodies are produced only when there are viable organisms in the central nervous system. Because the immunoblot test measures antibodies to many protein antigens, probably some antigens on immunoblots that are recognized by antibodies produced by infected horses may be valuable in diagnosis, but these antigens are not defined. The immunodominant surface antigen of *S. neurona*, named SnSAG1, has been characterized and is detected by antibodies from horses with clinical EPM from which organisms were recovered.[9]

A model of infection using *S. neurona* merozoites resulted in horses with clinical signs of EPM and a bank of true positive sera and CSF to evaluate a recombinant antigen ELISA (SAG1 ELISA) (paper under review). The serum and CSF from six horses before and after infection were used to determine the cut-off value to reliably differentiate between noninfection and infection in this assay. After establishing the test system based on the sera from the six infected horses, we examined sera from horses with a presumptive diagnosis of EPM and sera from Coggins (test negative for equine infectious anemia) samples from a normal population of horses.

MATERIALS AND METHODS

Production of Antigen

Recombinant SnSAG1 protein (rSAG1) was produced and purified from a GST expression vector cloned in *Escherichia coli* (Novagen, Madison WI) using previously described methods and manufacturer recommendations.[9] The total protein was determined using a BCA protein detection kit (Pierce, Rockford, IL). The recombinant protein was diluted to 10 μg/mL using carbonate buffer (Sigma, St. Louis MO).

SAG1 ELISA

NUNC Maxisorp plates (Nunc, Napeville, IL) were coated with recombinant protein by adding 50 μL rSAG1 (10 μg/mL) to each well of the 96-well plate and incubated overnight at 4°C. The plates were washed with PBST (phosphate buffered saline with Tween) (PBS, 0.05% Tween 20) and blocked using 1% BSA (bovine serum albumin) in PBST that was added to each well and incubated 30 min. at room temperature. Alternatively, plates were blocked with 1% gelatin (Sigma, St. Louis MO) in PBST. The plates were washed 3 times in PBST and either 50 μL serum or 50 μL CSF was added. The serum or CSF was diluted in dilution buffer. The plates were incubated 2 hours at room temperature and then washed three times with PBST. Fifty microliters of secondary antibody, antihorse IgG-alkaline phosphatase (Sigma, St. Louis MO) diluted 1:3000 in PBST was added to each well. The plates were incubated 1 hour and then washed three times with PBST. Freshly made substrate, para-nitrophenol phosphate (Sigma, St. Louis MO) was prepared in carbonate buffer (pH 9.6); 100 μL were added to each well; and it was incubated 45 minutes at room temperature. The plate was read using a Finstruments microplate reader equipped with a 405-nm filter.

To determine a reasonable cutoff for a positive test, set points for the SAG1 ELISA 330 serum and CSF samples were obtained from horses with a presumptive diagnosis of EPM. The serum samples were screened for anti-*S. neurona* antibodies at a dilution of 1:50, and CSF was screened undiluted. Serum and CSF were then serially diluted for titration using PBST containing 1% BSA. The set point was determined at a value so that sera that tested positive at 1:50 also tested positive at 1:100 were above the value while sera that were positive at 1:50 but negative at 1:100 fell below the value. It was determined from these data that an absorbance value greater or equal to 0.518 nm was considered a positive test result for both serum and CSF.

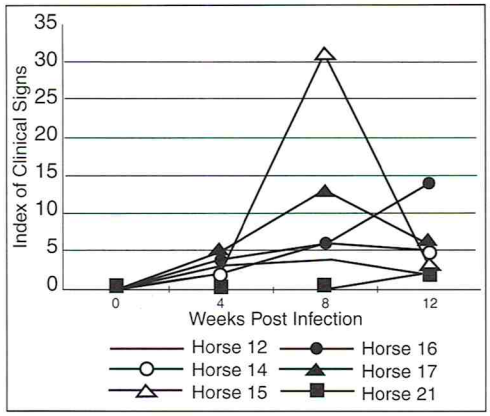

Figure 1. Clinical signs of horses pre and post infection.

The titer was recorded as the reciprocal of the last dilution with an absorbance value greater than or equal to 0.518.

Samples From Experimentally Infected Horses

Six clinically normal horses that were CSF *S. neurona* antibody negative by SAG1 ELISA were selected for infection using culture-derived merozoites of *S. neuorna*. The animals were anesthetized using Ketamine/Xylazine and CSF fluid removed via alanto-occipital tap using a 12-mL syringe fitted with a 3" spinal needle. The six horse were given 6,000 merozoites that had been incubated with homologous lymphocytes daily intravenously for 15 days. These horses ranged in age from 1 year to 20 years and included 5 mares and one gelding of various breeds (3 thoroughbreds, 2 quarter horses, and one mixed-breed mustang). Before infection, radiographs of the cervical spine were unremarkable for cervical spinal anomalies; serum was negative for equine viral arteritis; and vitamin E levels were within normal range.

All horses were evaluated neurologically by three independent blinded veterinarians four times during the study (preinfection and 4, 8, and 12 weeks after infection). The evaluations included observation for ataxia, lameness, and cranial nerve abnormalities. These observations were indexed (scores from three veterinarians added together) and are plotted in Figure 1. Samples of serum and CSF were collected from the six experimentally infected horses at each time, and are considered true positive samples for defining the parameters of diagnostic test systems. There were 48 total samples, of which 24 were serum samples and 24 were CSF samples. All samples were evaluated using the SAG1 ELISA and, for comparison, were submitted to Equine Biodiagnostics, (EBI, Lexington, KY) for immunoblot testing and CSF indices.

Fifteen neurologically normal horses housed on the same farm were neurologically and clinically normal and were not challenged. These animals were negative and remained negative when tested by SAG1 ELISA.

Additional samples were obtained from horses (1) with a presumptive diagnosis of EPM (n = 330) and (2) sera submitted for EIA (Coggins) testing (n = 147) submitted to a local diagnostic laboratory for sales testing. These serum samples were tested for anti-*S. neurona* antibodies (SAG1 ELISA) at a dilution of 1:50, and CSF samples were diluted 1:2 with PBST. The serum samples were then serially diluted with PBST containing 1% BSA to determine the endpoint titers.

Calculation of AI SAG1 Index

A high IgG_{index} (AI) to a specific antigen is supportive of IgG production in the CNS and may provide further support for a diagnosis of EPM in a horse that is positive for antibodies to *S. neurona*.[8] The SAG1 ELISA results were used to calculate a specific antibody index (AI_{SAG1}). The AI_{SAG1} for *S. neurona* was calculated from the formula AI_{SAG1}=AQ/Alb Q, in which AQ is antibody quotient and Alb Q is albumin quotient. The titer is the reciprocal of the last dilution with a positive $_{A405}$ and used in the formula AQ=(CSF titer)x 1000/serum titer, and AlbQ= (CSF alb/serum alb) x 1000.[8] Data used for this calculation are shown in Table 2.

RESULTS

Before infection, the six horses were neurologically and clinically normal. After infection, all

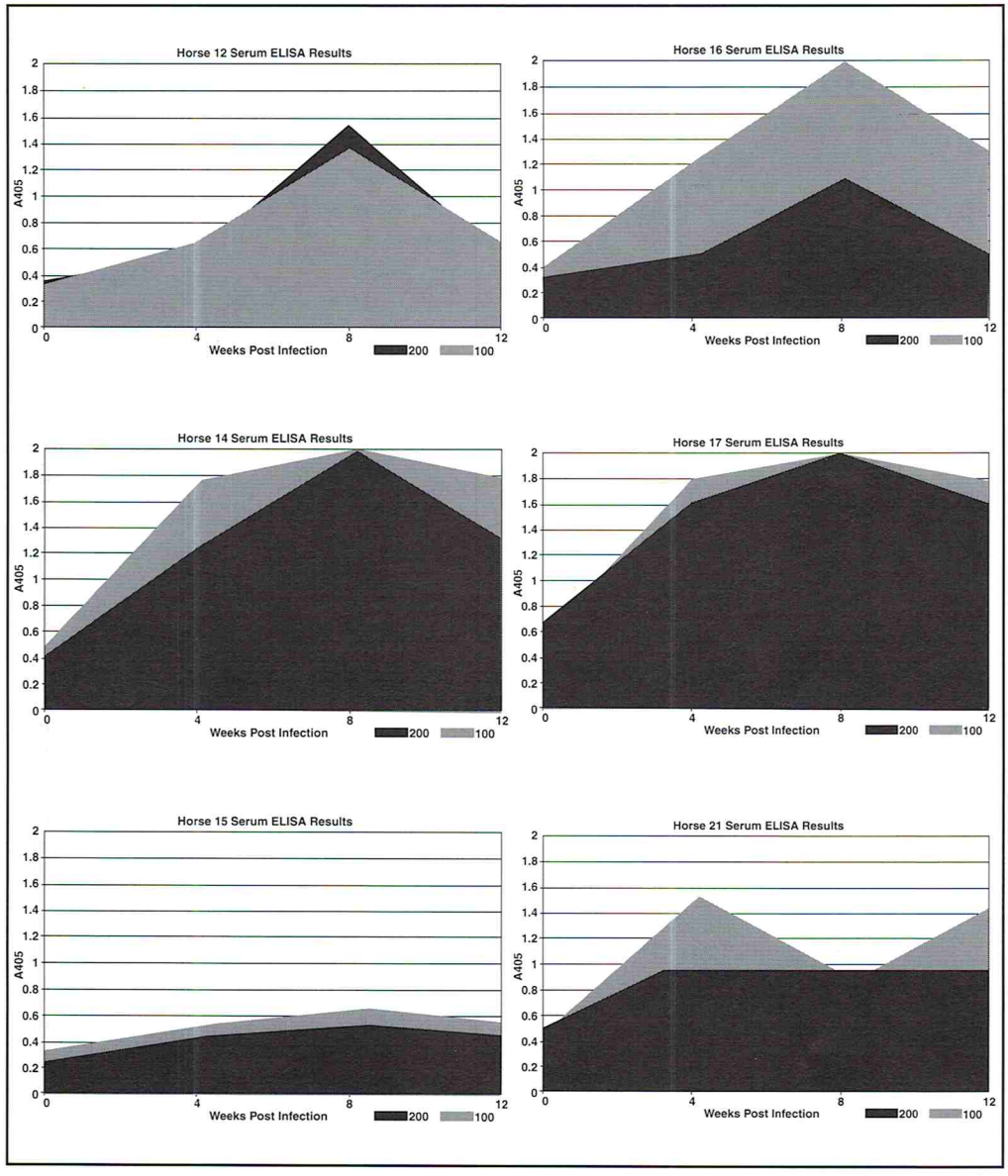

Figure 2. Graph of Serum ELISA results for 6 horses

horses showed sensory deficits over the neck, trunk, and rump; 3 horses were lame (horses 15, 16, and 17); 4 horses had various degrees of ataxia (horses 12, 15, 16, and 17), and one horse (horse 21) had minimal gait deficits but had muscle atrophy. Horse 14 showed numerous cranial nerve signs. A graph of the observed clinical signs is shown in Figure 1. Serum samples obtained before and after infection were tested using the *S. neurona* SAG1 ELISA at a dilution of 1:100 and 1:200 and are shown in Table 1. Comparison of the absorbance values for serum at selection and at 4, 8, and 12 weeks after infection (Fig. 2) showed that all animals seroconverted; however, there were individual differences in the responses. Five of the six horses had a twofold increase in titer at 4 weeks after infection, and one horse (horse 15) was serum-test positive at 100 by 8 weeks after infection. We detected an

Table 1. Table of A405 Results for Serum From 6 Experimentally Infected Horses

	Selection		4 WPI		8 WPI		12 WPI	
Screening Titer	**100**	**200**	**100**	**200**	**100**	**200**	**100**	**200**
ID								
12	0.319	0.349	0.627	0.535	1.34	1.53	0.627	0.535
14	0.473	0.407	1.76	1.27	2	2	1.76	1.27
15	0.31	0.218	0.498	0.418	0.622	0.506	0.498	0.418
16	0.428	0.377	1.31	0.528	2	1.14	1.31	0.528
17	0.551	0.653	1.79	1.6	2	2	1.79	1.6
21	0.456	0.447	1.55	0.963	0.89	0.931	1.55	0.963

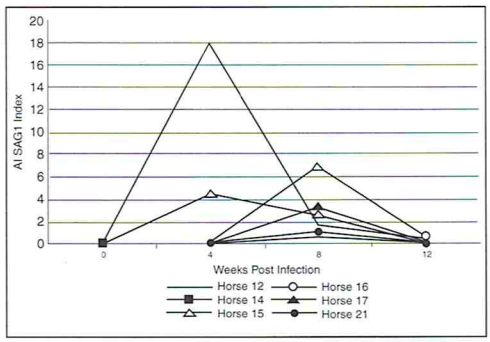

Figure 3. Graph of AISAG1 Antibody in the CSF

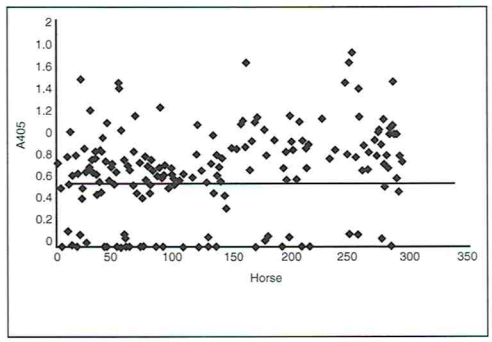

Figure 4. Scatter graph showing absorbance value of 330 samples obtained from horses with a presumptive diagnosis of EPM. The red line shows the cut off value of 0.518.

individual variation in the antibody response to infection. The antibody levels were significant by 4 weeks after infection. In this group of horses, a serum titer of 100 or greater coincided with clinical signs of EPM and clearly distinguished between uninfected and acutely infected horses.

The end point titer using the selected cut-off value was determined for three evaluations after infection and used to calculate the specific anti-*S. neurona* antibody (AI_{SAG1}) in the CSF (Table 2). These AI_{SAG1} CSF antibodies are more easily evaluated in graph format as represented in Figure 3. All horses were negative for antibodies in the CSF as determined by the SAG1 ELISA before infection and immunoconverted after infection. There were antibodies in the CSF detected in all horses at 8 weeks after infection. The CSF antibodies decreased by 12 weeks after infection in all horses. Antibodies detected in CSF by the SAG1 ELISA coincided with clinical signs in horses by 4 weeks after infection except number 21. In this case, CSF antibodies were a harbinger of clinical signs. The SAG1 ELISA clearly distinguished between uninfected and acutely infected horses when a CSF titer of 2 was used in experimentally infected horses.

To test the hypothesis that naturally infected horses have an immune response to SAG1, we tested 330 samples from horses with a presumptive diagnosis of EPM (Fig. 4). The SAG1 ELISA test results were positive in 85.4% of this population of horses when tested using 1:100 as a positive titer. A scatter graph of the 330 samples is shown in Figure 4.

To test the prevalence of antibodies to *S. neurona* in a random population of horses in Ocala, Florida, 147 sera obtained from samples submitted for Coggins testing were evaluated using the SAG1 ELISA. A summary of the results of these tests is presented in Table 3. When these serum samples were titrated to an endpoint, test results from this population showed that 76% of

Table 2. Values Used to Determine the AISAG1 in the CSF of 6 Experimentally Infected Horses

Horse ID	Serum IgG mg/dL	CSF IgG mg/dL	Serum Alb. mg/dL	CSF Alb. mg/dL	AQ	Serum Titer	CSF Titer	AI SAG1
0 DPC	12	2595	20.3	2812	43.9	1.6	≤50	0
0								
14	4229	15.9	3779	38.9	1	≤50	0	0
15	2185	9.3	3339	31.4	0.9	≤50	0	0
16	2810	9.3	3779	41.4	1.1	≤50	0	0
17	3144	14.5	3252	45.2	1.4	200	0	0
21	3031	23.5	2285	52.7	2.3	≤50	0	0
4 WPC								
12	2648	24.3	2812	54	1.9	200	2	4.46
14	4079	18.5	3164	37.7	1.2	200	0	0
15	1889	9.1	3076	47.7	1.6	200	0	0
16	2749	12.5	3955	37.7	1	200	2	17.92
17	4079	21	3867	45.2	1.2	400	0	0
21	5874	27.2	2724	32.6	1.2	400	1	0.15
8 WPC								
12	2648	24.3	2812	54	1.9	200	6	2.53
14	4079	18.5	3164	37.7	1.2	400	2	0.73
15	1889	9.1	3076	47.7	1.6	100	3	7.01
16	2749	12.5	3955	37.7	1	400	2	1.78
17	4079	21	3867	45.2	1.2	400	12	3.33
21	5874	27.2	2724	32.6	1.2	200	2	1.11
12 WPC								
12	3517	20.3	3603	54	1.5	200	2	0
14	4637	23.6	3603	40.2	1.1	1600	6	0.12
15	2514	12.6	3691	41.4	1.1	≤50	0	0.5
16	4063	11.7	3779	38.9	1	1600	8	0.49
17	3651	17.3	3164	46.5	1.5	1600	2	0.1
21	5087	22.9	2900	35.2	1.2	1600	1	0.06

animals have a titer of 50 or less and were considered negative, 18% have a titer of 100, and 6% have a titer of 200. It should be noted that these sera were collected before the availability of an EPM vaccine. Therefore, this would seem to indicate that up to 24% of normal horses are positive for *S. neurona* and are previously exposed or are potentially infected.

The mean A_{405} for the 147 random population serum samples was 0.430 at a 1:100 serum dilution (data not shown) while the average A_{405} for infected horses for the twelve weeks of the experiment was 1.32 at 1:100 and 1.04 at 1:200 (Table 1). We observed a higher A_{405} in infected horses at a 1:100 dilution than that obtained from random serum samples in the equine population at the same serum dilution. All infected horses immunoconverted against *S. neurona* in CSF by 8 weeks after infection.

The SAG1 ELISA and immunoblot (EBI, Lexington, KY) measure antibodies to different proteins; however, immunoblot data obtained on sera and CSF are shown in Table 4. Although immunoblot testing of CSF from horses without clinical signs of EPM have been shown to be of no value, the test was run to observe the test results after infection.[10] Using the CSF samples obtained

from the six experimentally infected horses as true-positive samples, 5 of the 6 horses were reported as positive by 8 weeks after infection. Horse 15, a horse that showed significant clinical signs of EPM, was reported as negative in the CSF at 4, 8, and 12 weeks after infection, and only 1 positive report was found from the serum sample at 4 weeks post infection. When comparing all samples with the SAG1 ELISA results, the EBI results reflected 17 of 18 serum samples and 15 of 18 CSF samples in agreement with the SAG1 ELISA.

DISCUSSION

An ideal diagnostic test would detect viable parasites in the horse before irreversible CNS pathology and thus allow successful drug treatment. What is known about the immune response in the horse has been determined using samples from horses suspected of EPM that represent chronic infections. The reproducible experimental induction of *S. neurona* infection in the horse may clarify some of the confusion interpreting the antibody response during acute and chronic infections.

The recombinant antigen, p29, recognized as an immunodominant surface antigen from *S. neurona,* and identified further as SAG1, was determined by molecular methods to be unique to *S. neurona* and shared between six isolates.[9] The recombinant antigen was tested against polyclonal antibodies raised in a rabbit against both *Toxoplasma gondii* and *Neospora*, other reported etiological agents of EPM in the horse.[11] Cross reactivity of antibodies raised against *T. gondii* and *Neospora* was detected when tested with merozoites of these species; however, no cross-reactivity of these antibodies to rSAG1 of *S. neurona* was found.[11] The SAG1 gene of *S. neurona* was expressed as a recombinant antigen and used in an ELISA test to detect antibodies against *S. neurona* in horses. Sera and CSF from naturally infected and experimentally infected horses contained antibodies to the recombinant protein after challenge infection. The SAG1 ELISA clearly distinguished between uninfected and acutely infected horses when a serum titer of 100 or a CSF titer of 2 was used in experimentally infected horses. Horses were infected with *S. neurona* and developed signs recognized as appropriate for horses with EPM using a merozoite model (unpublished observations). In a similar infection model, parasites were recovered by culture in 4 of 4 infected horses, relating clinical signs to *S. neurona* infection (unpublished observations). The merozoite model uses a method to infect horses that overcomes the as-yet undefined mechanisms of resistance to infection that has been shown in sporocyst challenge models. It is possible that cellular immune mechanisms contribute significantly to the pathogenesis of EPM and these mechanisms could moderate the specific humoral response in the horse. Despite the contribution of cellular immunity elicited during *S. neurona* infections in horses, the current study demonstrates the production of *S. neurona* specific antibody after acute infection that is detectable by SAG1 ELISA.

We have used the data generated from 6 experimentally infected horses to evaluate CSF and serum antibodies measured by SAG1 ELISA. We detected immunoconversion in serum and CSF that coincided with clinical signs in horses. Although an individual response in antibody production after infection occurred, measured by time of detection and degree of response, all horses became test positive. The presence of serum antibodies did not prevent infection, and a threefold increase in titer was observed after infection (horse 17). Using calculated values for CSF antibodies (AI_{SAG1}) a quantitative value could be applied to test results; however, serum testing yielded similar results and may be preferred due to the risk of CSF sampling. Paired serum samples run four weeks apart may have diagnostic value in field cases of suspect EPM.

We expect the antibodies measured to SAG1 by ELISA to differ from the pool of antibodies detected by immunoblot, and

therefore it is difficult to compare results between the two tests. The immunoblot detects antibodies to *S. neurona* proteins separated by reducing SDS polyacrylamide gel electrophoresis (PAGE). Reducing conditions used with SDS PAGE will decrease the amount of SAG1 detected by immunoblot. Despite the reduction of antibody binding to the SAG1 shown to occur under reducing conditions, monoclonal antibodies (mAb) specific to SAG1 of *S. neurona* run by EBI under their conditions visibly bind protein at the appropriate band.[9] The composite proteins considered in the subjective assessment of a positive immunoblot test result differ from the detection of antibody binding to one specific protein of *S. neurona* determined by SAG1 ELISA. Therefore, antibody binding to p29, if considered solely as the criteria for a positive test result, detected by immunoblot may allow a valid comparison of immunoblot and SAG1 ELISA. But Ellison demonstrated that the protein band seen on single dimension electrophoresis contained more than one protein and the co-migrating band has been identified by Howe et al.[11] as SnSAG4, a surface-associated membrane protein. Potential differences in polyclonal antibody binding to p29, shown on single-dimension immunoblots, may be attributable to the binding of two proteins rather than that of only SAG1, therefore confounding meaningful comparison of the tests. Furthermore, different dilutions of serum and CSF are used in the tests. It is unclear what method is used to discern a "weak positive" and a "low positive"; however, we deferred to the current opinion of EBI that a "weak positive" is considered negative (personal communication). The selection and production of the immunodominant *S. neurona* protein for use in the SAG1 ELISA probably results in a more-specific serologic test. Despite differences in sample dilution, conformation of protein because of reducing conditions, and test result evaluations, we found that after infection the immunoblot detected infection in five of the six horses evaluated, and the ELISA detected infection in all the infected horses. The most variation between the tests occurred prior to infection and at 4 weeks after infection. This observation is probably due to false-positive interpretation of immunoblot results before infection (neurologically normal horses) and the ability of the SAG1 ELISA to detect lower levels of antibodies produced to immunodominant antigens during early infections. The horses were selected based on no clinical evidence of neurologic disease and absence of antibodies in the CSF detected by ELISA.

The results of testing a random population of horses in Florida (n = 147) by SAG1 ELISA have shown that 18% have a titer of 100 and 6% have a titer of 200, accounting for the 24% positive test results. The neurologic status of this test population is unknown because samples were anonymous, obtained from serum submitted for Coggins testing. The numbers of positive test results obtained by SAG1 ELISA are significantly less than the positive test results reported in surveys using immunoblot testing, the level of anti-*S. neurona* detected by immunoblot exceed 50%.[4] Although the levels of antibodies detected in the normal population of horses in Ocala, Florida, may be geographical, it is most likely the difference is that the test used to measure these antibodies is more specific.

Because the SAG1 ELISA is unique to *S. neurona,* a specific antibody index could be calculated and used to quantitatively determine the amount of antibody in CSF fluid that is due to intrathecal production of antibodies that recognize SAG1 of S. *neurona*. In the acute phase of disease, we found that AI_{SAG1} was useful. We were able to follow antibody levels before infection through the acute stage of disease that was observed clinically. The determination of an AI was useful and further differentiated between consecutive ELISA test result titers. These data were obtained when a titer was falling and the horse was improving and indicated clinical improvement. This is in

Table 4. Immunoblot Data for Serum and CSF From Infected Horses

Serum	0 DPI (Selection)	4 WPI	8 WPI	12 WPI
12	Positive	Low Positive	Positive	Positive
14	Positive	Low Positive	Low Positive	Positive
15	Negative	Positive	Negative	Negative
16	Positive	Positive	Positive	Low Positive
17	Positive	Low Positive	Positive	Positive
21	Positive	Positive	Positive	Positive
CSF				
12	Low Positive	Positive	Positive	Positive
14	Negative	Positive	Positive	Low Positive
15	Negative	Negative	Negative	Negative
16	Negative	Positive	Positive	Low Positive
17	Low Positive	Low Positive	Positive	Positive
21	Low Positive	Positive	Positive	Positive

contrast to other studies, but those studies may not have examined acute cases of EPM.[8] Similar to other studies using AI_{index}, we detected a decrease in antibody production over the observation period.[8] Although the former studies attributed a decrease in AI_{SAG1} to treatment and found no predictive value for outcome, we observed a decrease in AI_{SAG1} without treatment that did predict outcome. It is possible that the decrease in AI_{SAG1} is in response to effective cellular immunity in the horse, evidence that the horse can, in some cases, effectively fight the infection. What is desired is a method of detecting acute *S. neurona* infection and facilitating natural elimination of the parasite with treatment before irreversible CNS damage.

Many factors are important in EPM, notably the contribution of time and inflammation to the course of the disease. Interestingly, the presence of serum, or in some cases CSF antibodies, that were reported by EBI immunoblot test results before infection were not protective against the infection of horses by this infection model. All available strains of *S. neurona* tested express SAG1; however, it has yet to be determined if there are strains of this parasite that infect horses but do not express SAG1. More data using all strains of *S. neurona* isolated from the CNS of horses will be required to determine immune differences detected by SAG1 ELISA.

In the experimentally infected animals, five horses had serum antibody titers that remained elevated for the duration of the study when tested by SAG1 ELISA. It is impossible to evaluate the significance of the elevated serum antibodies and clinical signs in this study because horses with high titers ($\geq$ 1600) were not followed until the antibodies decreased. However, factors that could contribute to the observed antibody levels are the method of infection, dose of merozoites used for the infection, establishment of a patent infection, and as-yet unidentified factors that modulate infection in the horse. As seen in other challenge experiments, the clinical signs did plateau in some animals (horses 15 and 21), and the CSF antibody titer or AI_{SAG1} could predict the clinical improvement. In two cases, the serum and CSF antibodies remained elevated and it is undetermined if these horses would succumb to further disease if held longer. Serum SAG1 ELISA was useful during the evaluation period when serial samples were used for the evaluation. Notably, we detected immunoconversion in the CSF and a rise in serum titer after acute infection measured by SAG1 ELISA.

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