Enzyme-Linked Immunosorbent Assays for Detection of Equine Antibodies Specific to Sarcocystis neurona Surface Antigens†

Jessica S. Hoane,1 Jennifer K. Morrow,2 William J. Saville,3 J. P. Dubey,4 David E. Granstrom,3 and Daniel K. Howe1*

Department of Veterinary Science, University of Kentucky, 108 Gluck Equine Research Center, Lexington, Kentucky 40546-00991; Equine Biodiagnostics/IDEXX, 1501 Bull Lea Road, Suite 104, Lexington, Kentucky 405112; Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1900 Coffey Road, Columbus, Ohio 43210-10923; and United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Beltsville, Maryland 20705-23504

Received 17 March 2005/Returned for modification 25 May 2005/Accepted 10 June 2005

Sarcocystis neurona is the primary causative agent of equine protozoal myeloencephalitis (EPM), a common neurologic disease of horses in the Americas. We have developed a set of enzyme-linked immunosorbent assays (ELISAs) based on the four major surface antigens of S. neurona (SnSAGs) to analyze the equine antibody response to S. neurona. The SnSAG ELISAs were optimized and standardized with a sample set of 36 equine sera that had been characterized by Western blotting against total S. neurona parasite antigens, the current gold standard for S. neurona serology. The recombinant SnSAG2 (rSnSAG2) ELISA showed the highest sensitivity and specificity at 95.5% and 92.9%, respectively. In contrast, only 68.2% sensitivity and 71.4% specificity were achieved with the rSnSAG1 ELISA, indicating that this antigen may not be a reliable serological marker for analyzing antibodies against S. neurona in horses. Importantly, the ELISA antigens did not show cross-reactivity with antisera to Sarcocystis fayeri or Neospora hughesi, two other equine parasites. The accuracy and reliability exhibited by the SnSAG ELISAs suggest that these assays will be valuable tools for examining the equine immune response against S. neurona infection, which may help in understanding the pathology of this accidental parasite-host interaction. Moreover, with modification and further investigation, the SnSAG ELISAs have potential for use as immunodiagnostic tests to aid in the identification of horses infected by EPM.

Sarcocystis neurona is a coccidian parasite that can infect horses and occasionally cause the neurologic disease equine protozoal myeloencephalitis (EPM) (6, 9). Horses become infected with S. neurona by ingesting sporocyst-contaminated food and water sources (8, 15). Ultimately, S. neurona can invade the central nervous system of the infected horse, causing focal or multifocal inflammation and EPM. S. neurona infection in horses is assessed by the detection of antibodies against the parasite in either the serum or cerebrospinal fluid (CSF); however, not all horses that seroconvert to S. neurona will develop EPM (9, 27). The seroprevalence of S. neurona infection in horses in the United States ranges between 0 and 89.2%, depending upon geographic locale (1–3, 10, 34, 37, 39). In contrast, the incidence of clinical EPM has been estimated at <1% (28). It is not well understood what factors are responsible for the dichotomy between apparent infection and clinical disease, but this ambiguity creates a major hindrance to EPM diagnosis and disease control.

Current technologies for detecting S. neurona antibodies in equine serum and CSF samples include Western blotting (17), a modified version of Western blotting (35), an S. neurona direct-agglutination test (SAT) (25), and an indirect fluorescent-antibody test (5). Each of these current serodiagnostic assays utilizes complete S. neurona merozoite preparations as the antigen source, which has several drawbacks. Specifically, propagation of parasite cultures is relatively time-consuming and expensive, and the use of whole-parasite preparations can increase the risk of false-positive results due to cross-reactivity with closely related pathogens, such as Sarcocystis fayeri (11, 38). Additionally, the current assays are not very amenable to quantitation, and their results can be subject to interpretation (16, 32). Given these shortcomings, a detailed and in-depth characterization of equine humoral responses to S. neurona infection is not feasible with the existing serologic tests.

Four related surface antigens have been identified in S. neurona merozoites, and these have been designated SnSAG1, SnSAG2, SnSAG3, and SnSAG4 (13, 20). To develop better tools for analyzing antibody responses to S. neurona infection, antibody capture enzyme-linked immunosorbent assays (ELISAs) were designed to utilize recombinant forms of the four S. neurona surface antigens (rSnSAGs). Comparison of the rSnSAG ELISAs with Western blot analysis of S. neurona merozoites confirmed that three of these assays are highly accurate and reliable. These ELISAs will serve as valuable tools for the evaluation of the equine humoral immune response to S. neurona infection, which may in turn allow discrimination between horses with EPM and those with asymptomatic S. neurona infections.

MATERIALS AND METHODS

Parasite culture. The SN3 strain of S. neurona and the Oregon strain of Neospora hughesi (7, 18) were maintained by serial passage in bovine turbinate cell monolayers. Upon lysis of the host cell monolayer, zoites were passed twice...
through 20-gauge (20-G), 22-G, and 25-G needles and filtered through a 3.0-μm Nucleopore (Whatman) membrane to remove host cell debris. The harvested parasites were counted with a hemocytometer, washed with phosphate-buffered saline (PBS), and stored at −20°C.

Recombinant-protein preparation. The four SnSAGs were expressed as recombinant proteins and purified by nickel column chromatography, as described previously (20). The concentration of the purified protein was determined by a colorimetric assay (Coomassie Plus Protein Assay Reagent; Pierce). Purified rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4 were each diluted in elute buffer (0.5 M NaCl and 20 mM Tris-HCl) without urea to final protein concentrations of 8.15 μg/ml, 23.0 μg/ml, 14.56 μg/ml, and 10.3 μg/ml, respectively.

Serum and CSF samples. The positive control serum samples were from two clinically affected horses that had histologically confirmed EPM. The negative control sample for all assays was a preinfection serum sample from a weanling used in an S. neurona infection trial (14). Thirty-six equine sera submitted to Equine Diagnostics (EBI)/IDEXX for S. neurona serology testing were used for standardization of the rSnSAG ELISAs. These samples had been classified as positive or negative in S. neurona Western blots using criteria established by EBI/IDEXX (9, 10). Samples from 27 EPM-confirmed horses were from a collection compiled at the University of Kentucky Gluck Equine Research Center. All cases were confirmed by histological examination of central nervous system tissue for the presence of lesions consistent with EPM, and prior Western blot analyses had demonstrated that all 27 horses had CSF antibodies against S. neurona. Paired serum and CSF samples were available for most EPM horses, with the exception of numbers 2, 5, 6, 14, and 16, for which CSF samples were absent, and horse 27, for which serum was absent. Three equine serum samples from a foi challenge trial (38) were used to examine assay cross-reactivity. An N. hughesi positive control serum sample was taken from a horse that was naturally infected with N. hughesi (30) and was provided by A. E. Marsh, Ohio State University.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Native or recombinant proteins were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer supplemented with protease inhibitor cocktail (Sigma) and separated on 12% polyacrylamide gels (24). For Western blot analysis, proteins were transferred to nitrocellulose membranes by semidy electrophoresis in Tris-glycine buffer, pH 8.3. Membranes were blocked with PBS containing nonfat dry milk, 0.1% Tween 20, and 5% normal goat serum (NGS), after which the membranes were incubated for 1 hour in primary antibody solution. The membranes were washed, followed by incubation for 45 minutes with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Membranes were processed for chemiluminescent detection using SuperSignal substrate (Pierce) and exposed to radiographic film or documented with a FluorChem 8800 imaging system (Alpha Innotech, Corp.).

ELISA. The rNhSAG1 ELISA was performed as described previously (19). For rSnSAG ELISAs, high-binding 96-well plates (Corning) were incubated overnight at 4°C with 100 μl of purified rSnSAG1, rSnSAG2, rSnSAG3, or rSnSAG4 diluted to 0.20 μg/ml, 1.00 μg/ml, 0.09 μg/ml, and 0.21 μg/ml, respectively. The plates were rinsed three times with PBS containing 0.05% Tween 20 (PBST) and blocked for 1.5 hours at room temperature (RT) with PBS containing 1% Tween 20, 5% NGS, and 0.01 g/ml nonfat dry milk. Primary sera or CSF was diluted to the appropriate concentration in PBS containing 0.1% Tween 20, 0.5% NGS, and 0.001 g/ml nonfat dry milk. One hundred-microliter aliquots of the antibody mixtures were added to duplicate wells of the plate and incubated for 2 hours at RT. The wells were rinsed five times with PBST and incubated for 2 hours at RT with 150 μl of horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (IgG) secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted to 1:10,000 in PBS containing 0.1% Tween 20, 0.5% NGS, and 0.001 g/ml nonfat dry milk. One hundred-microliter aliquots of the chromogenic substrate o-phenylenediamine dihydrochloride (Sigma) was dissolved in 0.5 M phosphate-citrate buffer to a concentration of 0.4 mg/ml, and 200 μl of substrate solution was added to each well. After a 10-minute incubation, the reaction was stopped with 50 μl of 3 M H2SO4, and the optical density at 490 nm (OD490) was measured in an Emax microplate reader (Molecular Devices). To account for interplate variation, the OD of each serum sample was expressed as a percentage of the high positive standard on the plate, calculated as described in the following equation (41): PP(%) = [OD(sample) – OD(NC)]/[OD(PC) – OD(NC)] × 100, where PP is the percent positivity of each sample. NC is the average negative control OD, and PC is the average positive control OD.

RESULTS

Recombinant-protein antibody recognition by Western blotting. Equine antibody reactivity with the four rSnSAGs was initially analyzed in Western blots and compared to Western blot results for S. neurona whole-parasite antigen. As shown in Fig. 1, CSF from five seropositive horses showed antibody recognition of rSnSAG2, whereas two seronegative horses did not react to the recombinant antigen. Importantly, there was no reactivity to other proteins that might be minor contaminants of the purified antigen preparation. Similar results were obtained with rSnSAG1, rSnSAG3, and rSnSAG4 (data not shown).

Standardization of rSnSAG ELISAs. Initially, checkerboard titrations were used to determine the optimal recombinant-protein concentrations and secondary-antibody dilutions for each rSnSAG ELISA. Under these defined conditions, significant background occurred with primary serum dilutions of less than 1:250, so this was selected as the starting dilution for the four assays. To standardize the ELISAs, total serum IgG antibody reactivity to each of the four surface antigens was determined on a sample set of 36 horse sera that had been previously characterized by Western blotting. Samples were tested with the ELISAs at a 1:250 dilution, and the mean OD490 value for each sample was converted into a PP value. To determine the optimal cutoff for each ELISA, sensitivity and

FIG. 1. Parallel Western blot analyses of (A) whole reduced S. neurona lysate (5 × 10^9 merozoites) and (B) 0.8 μg rSnSAG2 protein showed good correlation between CSF antibody recognition of the rSnSAG2 and native parasite antigen. The immunodominant antigens representing SnSAG1, SnSAG4, and SnSAG2 are indicated, as described previously (20). With the Western blot conditions used, native SnSAG3 was not visible. CSF samples were run at 1:25. +, confirmed EPM positive; −, confirmed EPM negative.
specificity were calculated at different PP values by comparison to Western blot results for the 36 samples (Table 1). Based on these evaluations, the rSnSAG2 ELISA showed the highest accuracy of 95.5% sensitivity and 92.9% specificity when a PP cutoff of 20% was used. In contrast, only 68.2% sensitivity and 71.4% specificity were achieved with the rSnSAG1 ELISA at its optimum PP cutoff of 10%. A PP of 15% was selected as the optimal cutoff for both the rSnSAG3 and the rSnSAG4 ELISAs (Table 1).

**Analysis of EPM-confirmed serum samples.** To investigate the total IgG serum antibody response in clinically affected horses, 26 samples from EPM-confirmed horses were tested in twofold dilutions from 1:250 to 1:8,000, and the previously defined optimal cutoff value for each rSnSAG ELISA was utilized to determine the end point titer for each sample. Serum antibody titers against rSnSAG4 were detected in 25 of the 26 (96.2%) EPM horses (Fig. 2). The rSnSAG2 and rSnSAG3 ELISAs yielded seropositive results in 24 of 26 (92.3%) serum samples. Only 18 of the 26 (69.2%) EPM horses had detectable serum antibody titers against rSnSAG1. Interestingly, some serum samples exhibited considerable variation in the antibody titer detected against each of the rSnSAGs (Fig. 2), suggesting that individual animals may generate unequal responses to the different *S. neurona* surface antigens.

| PP | Sensitivity/specificity (%)<sup>a</sup> |
|----|----------------------------------------|
|    | rSnSAG1 | rSnSAG2 | rSnSAG3 | rSnSAG4 |
| 5  | 68.2/50 | 100/14.3 | 100/14.3 | 100/14.3 |
| 10 | 68.2/71.4 | 100/57.1 | 90.9/42.6 | 100/50  |
| 15 | 54.5/85.7 | 95.5/78.6 | 90.9/78.6 | 95.5/85.7 |
| 20 | 45.5/92.9 | 95.5/92.9 | 77.3/85.7 | 90.9/85.7 |
| 25 | 45.5/100 | 90.9/100 | 72.7/100 | 90.9/85.7 |
| 30 | 45.5/100 | 81.8/100 | 63.6/100 | 86.4/100 |
| 35 | 40.9/100 | 72.7/100 | 54.5/100 | 81.8/100 |

<sup>a</sup> Calculations based on comparison to Western blot results for the 36 serum samples used for standardization of the ELISAs.
Analysis of EPM-confirmed CSF samples. To evaluate the total IgG antibody response in the CSF of clinically affected horses, CSF samples from 22 of the EPM-confirmed horses and four seronegative horses were tested at multiple dilutions against the four surface antigens. Due to limited CSF quantities, the samples were initially tested at dilutions of 1:12.5, 1:50, and 1:200. Samples that did not have a detectable antibody titer at the 1:12.5 dilution were subsequently retested at 1:2, 1:4, and 1:8 dilutions. Any sample at the 1:2 dilution with a PP greater than or equal to the defined cutoff for each rSnSAG ELISA was considered antibody positive. In total, 18 (81.8%), 18 (81.8%), 20 (90.9%), and 21 (95.5%) of the 22 CSF samples had detectable antibody titers against rSnSAG1, rSnSAG2, rSnSAG3, and rSnSAG4, respectively (Fig. 3). One negative control sample (horse n2) exhibited antibody reactivity to rSnSAG3 at the 1:2 dilution, but this sample was negative to rSnSAG1, rSnSAG2, and rSnSAG4. With the exception of five horses, very low CSF antibody titers against rSnSAG2 were observed. In contrast, CSF antibody titers were generally higher against rSnSAG1, rSnSAG3, and rSnSAG4. Similar to the serum results, considerable variation in the antibody titer to each of the four SnSAGs was observed in some samples (Fig. 3).

Assessment of antibody cross-reactivity. To determine whether the rSnSAGs cross-react with antibodies against closely related apicomplexan parasites, serum samples from three horses challenged with S. fayeri were tested in the ELISAs (38). A total of 13 serum samples were taken weekly from each horse during the duration of the trial. Although interpretation for S. neurona-specific serology was hindered (38), Western blot analysis clearly demonstrated that S. fayeri-infected horses produce antibodies that cross-react with multiple S. neurona antigens (Fig. 4A). When tested with the ELISAs at a 1:250 dilution, the serum samples exhibited no consistent or appreciable rise in antibody reactivity to the rSnSAGs over the course of the experiment (Fig. 4B). Although the calculated PP values occasionally equaled or eclipsed the cutoffs at one or a few time points, the antibody
reactivity was generally below the defined cutoffs for each of the assays.

Two of the 36 validation samples, numbers 27 and 33, were seropositive to *N. hughesi* by both ELISA and Western blotting (19). However, neither of these horses had a significant serologic response to the rSnSAGs (Fig. 1). Additionally, the serum and CSF from a horse that had been naturally infected with *N. hughesi* (29) were tested in the rSnSAG ELISAs. Previously, this horse had exhibited slight seroreactivity to *S. neurona* by Western blotting, suggesting prior exposure to the parasite (29). The ELISA analyses demonstrated that this horse had a serum titer of 1:500 to rSnSAG2 and 1:250 to rSnSAG4 (data not shown), which are consistent with the Western blot results. However, the horse was seronegative to the other two rSnSAGs and showed no CSF antibody reactivity to any of the rSnSAGs (Fig. 3, horse n4). Collectively, these results indicated that antibodies against two related equine pathogens, *S. fayeri* and *N. hughesi*, will not confound the rSnSAG ELISA results.

**DISCUSSION**

There is substantial precedent for utilizing the surface antigens of apicomplexan parasites in sensitive serodiagnostic tests (4, 19, 21, 23, 36). In this study, we developed ELISAs based on the immunodominant surface antigens of *S. neurona* (20), and these assays have proven accurate for detecting antibodies to *S. neurona* in equine sera and CSF. Importantly, the assays do not cross-react with antisera against *S. fayeri*, a closely related species that utilizes the horse as a natural intermediate host (11). The ELISA offers many advantages over current *S. neurona* serologic assays, including ease of use, high sample throughput, and more objective interpretation of results. Furthermore, the use of recombinant antigens in the ELISAs precludes the need for propagation of parasites in tissue cultures. The rSnSAG2, rSnSAG3, and rSnSAG4 ELISAs, in particular, will be important tools for the in-depth examination of the equine humoral response to *S. neurona* infection. With some modifications, such as combining SnSAGs in a single ELISA, and with further investigation using larger serum sets with more negative samples, these assays may also prove useful as serodiagnostic tests, as they showed high sensitivity and specificity compared to both Western blot serodiagnosis and postmortem diagnosis.

A variety of serologic assays have been developed to test for the presence of anti-*S. neurona* antibodies in serum or CSF. These include an SAT (25), which assesses antibody agglutination of formalin-fixed *S. neurona* merozoites, and an indirect fluorescent-antibody test (5), which monitors the presence of antibodies by fluorescence of merozoites. Both of these assays are well designed for testing large numbers of samples and determining end point titrations. The SAT has the added benefit of being useful for testing sera from other animal species.
without assay modification. Western blot analysis of total mer-
zoite protein, also referred to as the immunoblot assay, was
the first assay used for detecting serum and CSF antibodies
against *S. neurona*. Various permutations of the Western blot
assay have been reported and utilized (35), but the immuno-
blot assay as originally described remains the most commonly
used test for EPM immunodiagnosis (17). This assay was ini-
tially described in the early 1990s, and is based on antibody
recognition of low-molecular-weight antigens of unknown
identity (9, 10). The immunoblot assay provides a wealth of
information, thereby providing enhanced confidence in the
accuracy of the assay results. It is for this reason that Western
blotting is often used as the gold standard for serologic testing.

Unfortunately, there is an assortment of shared or unique
limitations that are associated with each of the serologic assays
described above. A number of these drawbacks are partially or
totally alleviated in the rSnSAG ELISAs. The above-men-
dioned need for culture-derived *S. neurona* merozoites is neg-
gated by using the rSnSAGs as the antigen source in these
ELISAs. Relative to propagation of *S. neurona* in tissue cul-
ture, production of these recombinant proteins is very simple
and inexpensive. For example, a 500-ml culture of bacteria
yielded 30 mg of rSnSAG2 protein, which is sufficient to test
greater than 100,000 serum or CSF samples in duplicate.
Additionally, the use of a specific defined antigen or epitope that
is known to be immunodominant reduces the likelihood of
cross-reactivity in serologic tests for other apicomplexan par-
asites (21, 23, 26, 33). Despite obvious antigenic cross-reactiv-
ity between *S. neurona* and *S. fayeri*, the rSnSAG ELISAs were
not confounded by the presence of anti-*S. fayeri* antibodies
(Fig. 4). Most significantly, the ELISA format utilizes an ob-
jective cutoff and is very amenable to modification for analysis
of specific antibody subsets (i.e., isotypes). Consequently, the
rSnSAG ELISAs will allow a more in-depth characterization of
the equine immune response to *S. neurona* infection.

One noteworthy and surprising result of these studies is that
serum antibody titers to rSnSAG1 could be detected in only
68.2% of the positive validation horses and 69.2% of the EPM
horses. Consistent with these results, some serum samples
tested by Western blotting at EBI/IDEXX fail to demonstrate
reactivity with the immunodominant 30-kDa antigen that is
primarily SnSAG1 (13, 20) despite reacting to multiple other
antigens, including those that are diagnostic (J. K. Morrow,
unpublished data). The prototypic SnSAG1 has been found to
be absent in cultured isolates of *S. neurona* from two EPM
horses (22, 31; D. K. Howe, *S. neurona* EST sequencing
project), and it is conceivable that this surface antigen poly-
morphism occurs in a proportion of *S. neurona* strains in
nature. Although a serodiagnostic assay has been developed
based on a recombinant form of SnSAG1 (12), our study indi-
cates that this antigen may not be the most reliable serolog-
ical marker for detecting *S. neurona* infection in horses. Cur-
rently, there is no evidence of strain variation at the other three
surface antigen loci of *S. neurona*. As such, SnSAG2, SnSAG3,
and SnSAG4 should serve as dependable markers for serologic
testing.

Elucidation of the factors that influence whether an *S. neu-
rona* infection will result in neurologic disease would greatly
enhance our understanding of EPM pathogenesis and the
identification of EPM-affected horses. The described ELISAs
will be useful for examining the equine humoral immune re-
response against specific parasite surface antigens during *S. neu-nona* infection. With the rSnSAG ELISAs, changes in antibody
titers can be easily followed throughout the course of infection
and disease progression. Also, studies using these assays are
under way to analyze specific antibody isotype responses
against the SnSAGs, thereby determining whether disparity in
humoral immune responses exists between horses that are se-
ropositive but clinically unaffected and horses that are afflicted
with EPM.

ACKNOWLEDGMENTS

We thank A. E. Marsh for providing the *N. hughesi*-positive serum
and CSF utilized in this study.

This research was supported by the Geoffrey C. Hughes Foundation
(J.S.H.) and grants from Fort Dodge Animal Health and the Amerman
Family Foundation (D.K.H.).

REFERENCES

1. Bentz, B. G., K. A. Eailey, J. Morrow, P. L. Claypool, and J. T. Saliki. 2003.
Seroprevalence of antibodies to *Sarcocystis neurona* in equids residing in
Oregon. J. Vet. Diagn. Invest. 15:597–600.

2. Bentz, B. G., D. E. Granstrom, and S. Stamper. 1997. Seroprevalence of
antibodies to *Sarcocystis neurona* in horses residing in horses residing in a county of southeast-
ern Pennsylvania. J. Am. Vet. Med. Assoc. 210:517–518.

3. Blythe, L. L., D. E. Granstrom, D. E. Hansen, L. L. Walker, J. Bartlett, and
S. Stamper. 1997. Seroprevalence of antibodies to *Sarcocystis neurona* in
horses residing in Oregon. J. Am. Vet. Med. Assoc. 210:525–527.

4. Chahah, B., I. Gaturaga, X. Huang, M. Liao, S. Fukimoto, H. Hirata, Y.
Nishikawa, H. Suzuki, C. Sugimoto, H. Nagasawa, K. Fukijski, I. Igarashi,
T. Mikami, and X. Xuan. 2003. Serodiagnosis of *Neospora caninum* infection
in cattle by enzyme-linked immunosorbent assay with recombinant truncated
SnSAG1. Vet. Parasitol. 118:517–518.

5. Duarte, P. C., B. M. Daft, P. A. Conrad, A. E. Packham, and I. A. Gardner.
2003. Comparison of serum indirect fluorescent antibody test with two West-
ern blot tests for the diagnosis of equine protozoal myeloencephalitis. J. Vet.
Diagn. Invest. 15:8–13.

6. Dubey, J. P., S. W. Davis, C. A. Speer, D. B. Bowman, A. de Lahuneta, D. E.
Granstrom, M. J. Topper, A. N. Hamir, J. F. Cummings, and M. M. Suter.
1991. *Sarcocystis neurona* n. sp. (Protozoa: Apicomplexa), the etiologic agent
of equine protozoal myeloencephalitis. J. Vet. Diagn. Invest. 3:176–178.

7. Dubey, J. P., S. Liddell, D. Mattson, C. A. Speer, D. K. Howe, and M. C.
Jenkins. 2001. Characterization of the Oregon isolate of *Neospora hughesi*
from a horse. J. Parasitol. 87:345–353.

8. Dubey, J. P., and D. S. Lindsay. 1998. Isolation in immunodeficient mice of
*Sarcocystis neurona* from opossum (*Didelphis virginiana*) feces, and its dif-
ferreration from *Sarcocystis falcatula*. Int. J. Parasitol. 28:1823–1828.

9. Dubey, J. P., D. S. Lindsay, W. J. Saville, S. M. Reed, D. E. Granstrom,
and C. A. Speer. 2001. A new *Sarcocystis neurona* and equine protozoal
myeloencephalitis (EPM). Vet. Parasitol. 98:89–131.

10. Dubey, J. P., S. M. Mitchell, J. K. Morrow, J. C. Rhyman, L. M. Stewart, D. E.
Granstrom, S. Romand, P. Thuilliez, W. J. Saville, and D. S. Lindsay. 2003.
Prevalence of antibodies to *Neospora caninum*, *Sarcocystis neurona*, and
Toxoplasma gondii in wild horses from central Wyoming. J. Parasitol. 89:
716–720.

11. Dubey, J. P., R. H. Streitel, P. C. Stromberg, and M. J. Toussant. 1977.
*Sarcocystis* fayer sp. n. from the horse. J. Parasitol. 63:443–447.

12. Ellison, S. P., T. J. Kennedy, and K. K. Brown. 2003. Development of an
ELISA to detect antibodies to *SAG1* in the horse. Int. J. App. Res. Vet.
Med. 1:318–327.

13. Ellison, S. P., A. L. Omara-Opyene, C. A. Yowell, A. E. Marsh, and J. B.
Dame. 2002. Molecular characterisation of a major 29 kDa surface antigen
of *Sarcocystis neurona*. Int. J. Parasitol. 32:217–225.

14. Fenger, C. K., D. E. Granstrom, A. A. Gajadhar, N. M. Williams, S. A.
McCruiff, S. Stamper, J. L. Langemeier, and J. P. Dubey. 1997. Experimen-
tial induction of equine protozoal myeloencephalitis in horses using *Sarcocystis*
sp. sporocysts from the opossum (*Didelphis virginiana*). Vet. Parasitol.
68:199–213.

15. Fenger, C. K., D. E. Granstrom, J. L. Langemeier, S. Stamper, J. M.
Donahue, J. S. Patterson, A. A. Gajadhar, J. V. Marteniuk, Z. Xiaoimin, and
J. P. Dubey. 1995. Identification of oocysts (*Didelphis virginiana*) as the
putative definitive host of *Sarcocystis neurona*. J. Parasitol. 81:916–919.

16. Furr, M. R., M. MacKay, D. Granstrom, H. Schott II, and F. Andrews.
2002. Clinical diagnosis of equine protozoal myeloencephalitis (EPM). J. Vet.
Intern. Med. 16:618–621.

17. Granstrom, D. E., J. P. Dubey, S. W. Davis, R. Fayer, J. C. Fox, K. B.

18. Granstrom, D. E., J. P. Dubey, S. W. Davis, R. Fayer, J. C. Fox, K. B.

19. Granstrom, D. E., J. P. Dubey, S. W. Davis, R. Fayer, J. C. Fox, K. B.
Poonacha, R. C. Giles, and P. F. Comer. 1993. Equine protozoal myeloencephalitis: antigen analysis of cultured Sarcocystis neurona merozoites. J. Vet. Diag. Investig. 5:88–90.

18. Hamir, A. N., S. J. Tornquist, T. C. Gerros, M. J. Topper, and J. P. Dubey. 1998. Neospora caninum-associated equine protozoal myeloencephalitis. Vet. Parasitol. 79:269–274.

19. Hoane, J. S., M. R. Yeevan, S. Stamper, W. J. A. Saville, J. K. Morrow, D. S. Lindsay, and D. K. Howe. 2005. Recombinant NhsAG1 ELISA: a sensitive and specific assay for detecting antibodies against Neospora hughesi in equine serum. J. Parasitol. 91:446–452.

20. Howe, D. K., R. Y. Gaji, M. Mroz-Barrett, M. J. Gubbelis, B. Striepen, and S. Stamper. 2005. Sarcocystis neurona merozoites express a family of immunogenic surface antigens that are orthologues of the Toxoplasma gondii surface antigens (SAGs) and SAG-related sequences. Infect. Immun. 73:1023–1033.

21. Howe, D. K., K. Tang, P. A. Conrad, K. Sverlow, J. P. Dubey, and L. D. Sibley. 2002. Sensitive and specific identification of Neospora caninum infection of cattle based on detection of serum antibodies to recombinant Ncp29. Clin. Diag. Lab. Immunol. 9:611–615.

22. Hyun, C., G. D. Gupta, and A. E. Marsh. 2003. Sequence comparison of Sarcocystis neurona surface antigen from multiple isolates. Vet. Parasitol. 112:11–20.

23. Kimbila, E. N., X. Xuan, X. Huang, T. Miyazawa, S. Fukumoto, M. Mishima, H. Suzuki, C. Sugimoto, H. Nagasawa, K. Fujisaki, N. Suzuki, T. Mikami, and I. Igarashi. 2001. Serodiagnosis of Toxoplasma gondii infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1. Vet. Parasitol. 102:35–44.

24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

25. Lindsay, D. S., and J. P. Dubey. 2001. Direct agglutination test for the detection of antibodies to Sarcocystis neurona in experimentally infected animals. Vet. Parasitol. 95:179–190.

26. Louie, K., K. W. Sverlow, B. C. Barr, M. L. Anderson, and P. A. Conrad. 1997. Cloning and characterization of two recombinant Neospora protein fragments and their use in serodiagnosis of bovine neosporosis. Clin. Diag. Lab. Immunol. 4:692–699.

27. Mackay, R. J. 1997. Equine protozoal myeloencephalitis. Vet. Clin. N. Am. Equine Pract. 13:79–96.

28. Mackay, R. J., D. E. Granstrom, W. J. Saville, and S. M. Reed. 2000. Equine protozoal myeloencephalitis. Vet. Clin. N. Am. Equine Pract. 16:405–425.

29. Marsh, A. E., B. C. Barr, J. Madigan, J. Lakritz, R. Nordhausen, and P. A. Conrad. 1996. Neosporosis as a cause of equine protozoal myeloencephalitis. J. Am. Vet. Med. Assoc. 209:1907–1913.

30. Marsh, A. E., B. C. Barr, A. E. Packham, and P. A. Conrad. 1998. Description of a new Neospora species (Protozoa: Apicomplexa: Sarcocystidae). J. Parasitol. 84:983–991.

31. Marsh, A. E., P. J. Johnson, J. Ramos-Vara, and G. C. Johnson. 2001. Characterization of a Sarcocystis neurona isolate from a Missouri horse with equine protozoal myeloencephalitis. Vet. Parasitol. 95:143–154.

32. Morley, P. S., and W. J. A. Saville. 1997. Equine protozoal myeloencephalitis: what does a positive test mean? Am. Assoc. Equine Pract. Ann. Proc. 43:1–5.

33. Nishikawa, Y., Y. Kousaka, K. Tragoolpua, X. Xuan, L. Makala, K. Fujisaki, T. Mikami, and H. Nagasawa. 2001. Characterization of Neospora caninum surface protein NcSRS2 based on baculovirus expression system and its application for serodiagnosis of Neospora infection. J. Clin. Microbiol. 39:3987–3991.

34. Rossano, M. G., J. B. Kaneene, B. D. Banks, H. C. Schott, and L. S. Mansfield. 2001. The seroprevalence of antibodies to Sarcocystis neurona in Michigan equids. Prev. Vet. Med. 48:113–128.

35. Rossano, M. G., L. S. Mansfield, J. B. Kaneene, A. J. Murphy, C. M. Brown, H. C. Schott II, and J. C. Fox. 2000. Improvement of Western blot test specificity for detecting equine serum antibodies to Sarcocystis neurona. J. Vet. Diag. Investig. 12:28–32.

36. Santoro, F., D. Alfilm, R. Pierce, J. Y. Cesbron, G. Ovalaque, and A. Capron. 1985. Serodiagnosis of Toxoplasma infection using a purified parasite protein (p530). Clin. Exp. Immunol. 62:262–269.

37. Saville, W. J., S. M. Reed, D. E. Granstrom, K. W. Hinchcliff, C. W. Kohn, T. E. Wittum, and S. Stamper. 1997. Seroprevalence of antibodies to Sarcocystis neurona in horses residing in Ohio. J. Am. Vet. Med. Assoc. 210:519–524.

38. Saville, W. J. A., J. P. Dubey, M. J. Oglesbee, C. D. Sofaly, A. E. Marsh, E. Elissur, M. C. Vianna, D. S. Lindsay, and S. M. Reed. 2004. Experimental infection of ponies with Sarcocystis fayeri and differentiation from Sarcocystis neurona infections in horses. J. Parasitol. 90:1487–1491.

39. Tillotson, K., P. M. McCue, D. E. Granstrom, D. A. Dargatz, M. O. Smith, and J. L. Traub-Dargatz. 1999. Seroprevalence of antibodies to Sarcocystis neurona in horses residing in northern Colorado. J. Equine Vet. Sci. 19:122–126.

40. Vardeleon, D., A. E. Marsh, J. G. Thorne, W. Loch, R. Young, and P. J. Johnson. 2001. Prevalence of Neospora hughesi and Sarcocystis neurona antibodies in horses from various geographical locations. Vet. Parasitol. 95:273–282.

41. Wright, P. F., E. Nilsson, E. M. VanRosij, M. Lelenta, and M. H. Jeggo. 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev. Sci. Technol. 12:435–450.