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ANTIGENIC EVALUATION OF A RECOMBINANT BACULOVIRUS-EXPRESSED *SARCOCYSTIS NEURONA* SAG1 ANTIGEN

G. D. Gupta, J. Lakritz*, W. J. Saville†, R. S. Livingston‡, J. P. Dubey§, J. R. Middleton‖, and A. E. Marsh†#
Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211. *e-mail: marsh.2061@osu.edu*

ABSTRACT: *Sarcocystis neurona* is the primary parasite associated with equine protozoal myeloencephalitis (EPM). This is a commonly diagnosed neurological disorder in the Americas that infects the central nervous system of horses. Current serologic assays utilize culture-derived parasites as antigen. This method requires large numbers of parasites to be grown in culture, which is labor intensive and time consuming. Also, a culture-derived whole-parasite preparation contains conserved antigens that could cross-react with antibodies against other *Sarcocystis* species and members of Sarcocystidae such as *Neospora* spp., *Hammondia* spp., and *Toxoplasma gondii*. Therefore, there is a need to develop an improved method for the detection of *S. neurona*–specific antibodies. The sera of infected horses react strongly to surface antigen 1 (SnSAG1), an ~29-kDa protein, in immunoblot analysis, suggesting that it is an immunodominant antigen. The *SnSAG1* gene of *S. neurona* was cloned, and recombinant *S. neurona* SAG1 protein (rSnSAG1-Bac) was expressed with the use of a baculovirus system. By immunoblot analysis, the rSnSAG1-Bac antigen detected antibodies to *S. neurona* from naturally infected and experimentally inoculated equids, cats, rabbit, mice, and skunk. This is the first report of a baculovirus-expressed recombinant *S. neurona* antigen being used to detect anti–*S. neurona* antibodies in a variety of host species.

Equine protozoal myeloencephalitis (EPM) is a well-recognized neurological disease of equids in North and South America caused primarily by *Sarcocystis neurona* (MacKay, 1997; Dubey, Lindsay et al., 2001). It causes asymmetric ataxia, muscle atrophy, and weakness, often necessitating killing of the affected horse (MacKay, 1997).

The opossum has been reported as the definitive host (Fenger et al., 1995, 1997) of this parasite, which sheds sporocysts in its feces. Other species such as the 9-banded armadillos (Cheadle, Tanhauser et al., 2001; Tanhauser et al., 2001), the striped skunk (Cheadle, Yowell et al., 2001), the cat (Dubey et al., 2000), and the raccoon (Dubey, Saville et al., 2001) develop sarcocysts and serve as intermediate hosts. The horse is believed to be an aberrant host for *S. neurona* because infectious sarcocysts have not been detected in their tissues. At some point during infection in the horse, *S. neurona* invades the brain and spinal cord, where the merozoite stage replicates and causes disease. The merozoite antigens are exposed to the host's immune system during the course of infection. Hence, most horses that have documented *S. neurona* by microscopic examination of their tissues have antibodies to the parasite.

EPM is diagnosed in horses by physical examination, ruling out other possible causes of neurologic disease, and cerebrospinal fluid (CSF) antibody evaluation (Cohen and MacKay, 1997). The sera of infected horses react strongly to the ~29-kDa protein in immunoblot analysis, suggesting that this is an immunodominant antigen (Liang et al., 1998; Marsh et al., 2001). Recently, Ellison et al. (2002) reported the sequence of the gene encoding this immunodominant surface antigen and named it *S. neurona* surface antigen 1 (*SnSAG1*) because of similarity in size with the *SAG1* gene of *Toxoplasma gondii*. The *T. gondii* p30 or TgSAG1 has undergone extensive evaluation as both diagnostic and vaccine antigen candidates (Nielsen et al., 1999; Haumont et al., 2000; Kimbita et al., 2001; Chen et al., 2002). Therefore, the SnSAG1 protein is a potential candidate as a diagnostic or subunit vaccine antigen for *S. neurona*. Currently, immunoblot analysis or indirect fluorescent antibody test (IFAT) are used for EPM diagnosis (Granstrom et al., 1993; Duarte et al., 2003). A major disadvantage of these tests is that they use whole-parasite preparations as antigen. This requires large numbers of culture-derived live parasites to produce sufficient parasite antigen, which is time consuming and labor intensive. Moreover, whole-parasite antigen preparations might contain epitopes conserved among other *Sarcocystis* spp. (Mayhew et al., 1978) and apicomplexans, which could result in cross-reactivity with antibodies directed to these parasites.

Because of the problems associated with a lack of specificity in the current testing methods, there is a need to improve testing by developing a *S. neurona*–specific diagnostic antigen. The goals of this study were to clone the *SnSAG1* gene, express and purify recombinant SnSAG1 antigen with a baculovirus expression system, and evaluate this protein for use as a diagnostic antigen to detect anti–*S. neurona* antibodies in animals naturally infected or experimentally inoculated with *S. neurona*.

MATERIALS AND METHODS

Construction of recombinant baculovirus

Sarcocystis neurona (SN-UCD1) was cultivated as previously described (Marsh et al., 1996). Total RNA was isolated from approximately 1×10^8 parasites with the Trizol reagent (GIBCO, Invitrogen Corporation, Carlsbad, California) according to the manufacturer's instructions and reverse transcribed by MuLV reverse transcriptase with the use of an oligo-dT primer (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, New Jersey).

The *S. neurona SAG1* gene was amplified by PCR with primers having a restriction site for *Eco*R1 or *Hin*dIII. Each 50-μl reaction mixture contained 1.25 U of *Taq* polymerase, 2 ng of cDNA as template, and primers ForBac (5′ GGT AAg aat tcG CGG TAA TGC TGC ATC ATT AGG 3′) and RevBac (5′ GCA CTG CAA GCT tCT AAC TGG 3′). The uppercase letters of the primers correspond to the *S. neurona* cDNA

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* Department of Large Animal Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210.
† Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210.
‡ Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211.
§ Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705.
‖ Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211.
To whom correspondence should be addressed.

sequence, whereas the lowercase letters represent engineered bases to create restriction endonuclease sites for *Eco*R1 or *Hin*dIII in the ForBac and RevBac primers, respectively. Thermocycling parameters consisted of an initial denaturation step (94 C, 5 min), followed by 30 cycles of denaturation (94 C, 30 sec), annealing (54 C, 30 sec), and extension (74 C, 105 sec). After amplification, a portion of the PCR mixture was analyzed on ethidium bromide–stained agarose gel.

The remaining reaction mixture was used to purify the desired PCR product from gel with the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California) and was digested with *Eco*R1 and *Hin*dIII. The Sn-SAG1 was directionally cloned, in frame, into the His-tagged fusion vector pFastBac HTa (Invitrogen, Carlsbad, California) to create the plasmid Sn-SAG1-pFastBac HTa. This insert was verified by vector sequence–specific primers with automated DNA sequencing. This plasmid was used to construct independent recombinant baculovirus expression vector Sn-SAG1-Bac with the Bac-to-Bac Baculovirus expression system (Invitrogen).

Production and purification of recombinant antigen

Standard insect cell and baculovirus propagation protocols were followed (Bernard et al., 2000). High-titer stock of recombinant baculovirus was generated in *Spodoptera frugiperda* (Sf-9) insect cells (Invitrogen), and recombinant antigen for diagnostic purposes was expressed in High-5 (Invitrogen) insect cells. Pilot experiments that used multiple replicates of T25 flasks were performed to determine the kinetics for maximal production of the recombinant protein. Individual transfected and uninfected cell flasks were monitored for growth and harvested daily between days 3 and 8 of transfection. During pilot expression study, day 5 postinfection was found to be the optimum time for extraction of the maximum quantity of recombinant antigen for insect cells with recombinant virus, compared with earlier time points that were tested with aliquots of the unpurified antigen and host cell controls and by immunoblot analysis with polyclonal rabbit antibodies against *S. neurona*.

To generate large quantities of recombinant *S. neurona* surface antigen 1 in baculovirus (rSnSAG1-Bac) for use as antigen, 10 T75 flasks were seeded with High-5 insect cells in Insect-Xpress medium (BioWhittaker, Inc., Walkersville, Maryland) supplemented with 5% fetal bovine serum. Cells were monitored with an inverted microscope and were infected with recombinant baculovirus. At 5 days postinoculation, the cells were pelleted by centrifugation at 500 *g* for 15 min at 4 C, washed twice with phosphate-buffered saline (PBS), resuspended in PBS containing proteinase inhibitor, and lysed by Insect Cell Lysis Buffer, pH 7.8 (10 mM Tris-Cl, 130 mM NaCl, 1% [v/v] Triton-X, 10 mM NaF, 10 mM Na_3PO_4, 10 mM sodium pyrophosphate, and 2 mM 4-[aminoethyl]-benzenesulfonyl fluoride hydrochloride). Insoluble material was pelleted at 40,000 *g* for 30 min at 4 C. The clarified supernatant (semipurified antigen) was mixed gently with Ni-NTA Resin (Invitrogen) overnight at 4 C. The His-tagged antigen was purified from the column by a linear gradient of imidazole. The recovered protein was concentrated following dialysis in PBS. Uninfected cells underwent similar harvesting procedures and immunoblotting to serve as a negative antigen control.

Immunoblot analysis

Five days after infection, semipurified rSnSAG1-Bac antigen from insect cells was evaluated on 4–12% Bis-Tris (Invitrogen) sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreduced and reduced conditions. Lysates of uninfected insect cells and *S. neurona* whole-parasite antigen lysates were used as negative and positive controls, respectively, under identical conditions. Molecular mass markers were included on each gel to determine approximate molecular size of the protein bands. The gels were either stained with SimpleBlue (Invitrogen) or transferred by electrophoresis to nitrocellulose membranes (Bio-Rad, Hercules, California). Membranes were blocked with 5% nonfat dried milk (NFDM) in Tris-buffered saline with 0.05% Tween 20 (TBS-Tween) for 1 hr and were probed with antibodies from the undiluted CSF of a histopathologically confirmed EPM horse (Marsh et al., 1996) or with *S. neurona*–specific monoclonal antibody 2A7-18 (Marsh et al., 2002) at 1:50 dilution as primary antibody for 1 hr at room temperature. The membranes were washed for 30 min in TBS-Tween and incubated with affinity-purified phosphatase-labeled goat anti-horse immunoglobulin (IgG; Kirkegaard and Perry Laboratories, Gaithersburg, Maryland), at 1:500 dilution, or affinity-purified peroxidase-labeled goat anti-mouse IgG (Kirkegaard and Perry Laboratories), at 1:10,000 dilution, in 1% NFDM in TBS-Tween for 2 hr. The membranes were washed for 30 min in TBS-Tween and developed in nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) alkaline-phosphatase substrate solution (Roche Diagnostic Corporation, Indianapolis, Indiana) or 3,3′-diaminobenzidine peroxidase substrate (Sigma, St. Louis, Missouri).

The column-purified recombinant antigen (rSnSAG1-Bac) was also subjected to the immunoblot procedure described previously under the same conditions to compare reactivity with semipurified recombinant antigen, whole-parasite lysate of *S. neurona*, and lysate of uninfected insect cells. It was found that column-purified recombinant antigen had reactivity similar to that seen with semipurified recombinant antigen. Hence, the semipurified recombinant antigen was used to analyze further the panel of antibodies from various species to detect *S. neurona* infection. The panel (Table I) included *S. neurona* whole parasite–inoculated polyclonal rabbit antibody (Marsh et al., 1996); sera (1:50) from 6 naturally infected EPM horses and 6 undiluted CSF samples from the same horses (Marsh et al., 1996, 2001); pre- and postimmunization sera (1:50) from 5 horses immunized with *S. neurona* whole-parasite vaccine (Fort Dodge Animal Health, Fort Dodge, Iowa) and 2 with media–adjuvant mixture as vaccine placebo; sera (1:50) from 17 horses (seronegative population) located on Jeju Island, South Korea (Gupta et al., 2002) and New Zealand (Vardeleon et al., 2001); pre and postimmunization sera (1:50) from 3 ponies experimentally inoculated with sporulated oocysts of *Sarcocystis fayeri*; pre and postimmunization serum (1:10) from a skunk experimentally inoculated with *S. neurona* (Cheadle, Yowell et al., 2001); and pre and postimmunization sera (1:1,500) from 5 cats experimentally inoculated with *S. neurona* (4 cats with *S. neurona* and 1 cat with host cells) (Butcher et al., 2002). The recombinant antigen was also evaluated for cross-reactivity with a 1:3,000 dilution of polyclonal antibodies against *Sarcocystis falcatula* (Dubey, Garner et al., 2001), *Neospora caninum* (Barr et al., 1994), *Neospora hughesi*, and *Hammondia* sp. (Romand et al., 1998) developed in experimentally inoculated rabbits. The recombinant antigen was also tested against a 1:50 dilution of serum from a pony experimentally inoculated with *T. gondii* oocysts (Dubey and Desmonts, 1987).

After probing for 1–2 hr in primary antibody, the membranes were washed for 30 min in TBS-Tween and incubated with the appropriate secondary antibody in 1% NFDM in TBS-Tween for 2 hr. Secondary antibodies used were affinity-purified phosphatase-labeled goat anti-horse IgG (Kirkegaard and Perry Laboratories) at 1:500 dilution for horse samples, peroxidase-conjugated affinipure mouse anti-rabbit IgG (Jackson Immunoresearch Laboratory, West Grove, Pennsylvania) at 1:5,000 dilution for rabbit samples, peroxidase-labeled affinity-purified goat anti-cat IgG (Kirkegaard and Perry Laboratories) at 1:2,000 for the cat samples, or affinity-purified peroxidase-labeled goat anti-raccoon IgG (Kirkegaard and Perry Laboratories) at 1:500 for the skunk samples. The immunoblots were developed in enhanced chemiluminescence western blot detection reagent (Amersham BioSciences, Piscataway, New Jersey) or NBT/BCIP solution for peroxidase- or phosphatase-labeled secondary antibody, respectively. The immunoblot banding pattern was compared between the whole-parasite lysate immunoblot and the immunoblots produced with the recombinant antigen. Previously confirmed positive and negative control sera, CSF, or a combination were included in each blot.

RESULTS

The size of purified product obtained after PCR amplification with ForBac and RevBac primers was approximately 1,300 bp. After restriction enzyme digestion of purified PCR product with *Eco*R1 and *Hin*dIII enzymes, the ~1,300-bp product remained intact, as observed with gel electrophoresis. The cut product was incorporated, in frame, in the bacmid system for expression of recombinant antigen as verified by automatic DNA sequencing results.

When the expressed recombinant antigen (semipurified or column purified) was evaluated by immunoblot, both monoclo-

TABLE I. List of samples evaluated.

Species	No. animals*	Reference
Equine		
EPM-confirmed horses	6	Marsh et al. (1996, 2001)
Vaccinated horses	5	Marsh (unpubl.)
Placebo horses	2	Marsh (unpubl.)
Jeju Island (South Korea) horses	10	Gupta et al. (2002)
New Zealand horses	7	Vardeleon et al. (2001)
Toxoplasma gondii–inoculated pony	1	Dubey and Desmonts (1987)
Neospora hughesi infected	1	Marsh et al. (1996)
Sarcocystis fayeri–inoculated ponies	3	Saville et al. (in press)
Nonequids		
Sarcocystis neurona–inoculated skunk	1	Cheadle, Yowell et al. (2001)
S. neurona–inoculated cats	5	Butcher et al. (2002)
Rabbit (*Sarcocystis falcatula*)†	1	Dubey, Garner et al. (2001)
Rabbit (*S. neurona*)†	1	Marsh et al. (1996)
Rabbit (*Neospora caninum*)†	1	Barr et al. (1994)
Rabbit (*Hammondia*)†	1	Romand et al. (1998)
Rabbit (*N. hughesi*)†	1	Marsh (unpubl.)
SnSAG1-specific monoclonal antibody 2A7-18	1	Marsh et al. (2002)

* Number of animals evaluated.
† Used for the production of high-titer polyclonal antibodies.

nal antibody 2A7-18 and antibodies in CSF of an EPM-positive horse recognized the nonreduced recombinant antigen, but not the reduced one. The nonreduced recombinant SnSAG1-Bac antigen was visualized as an ~31-kDa immunoreactive band, and the nonreduced SnSAG1 of *S. neurona* whole parasite was visualized as an ~29-kDa immunoreactive protein band. The larger molecular size of rSnSAG1-Bac (~31 kDa) was caused by additional amino acids from the bacmid expression vector. The uninfected insect cell lysate gave no visible banding, which signifies no nonspecific binding of the CSF to any of the insect cell antigens.

By immunoblot analysis, the rSnSAG1-Bac antigen (semipurified) reacted strongly as an ~31-kDa band with 4 (H1–H4) of 6 histopathologically confirmed, naturally infected EPM horses when both sera and CSF were tested (Fig. 1). The same panel of serum and CSF samples was also checked by immunoblot with culture-derived *S. neurona* whole parasites as antigen, in which 5 (H1–H5) of 6 EPM-positive sera per CSF sample reacted strongly as an ~29-kDa band (Fig. 2). The CSF and serum of the negative control (Fig. 1, lane NC) did not react with either of the 2 antigens.

All 17 serum samples of an *S. neurona* seronegative population from South Korean and New Zealand horses showed no reactivity with the recombinant SnSAG1-Bac antigen by immunoblot (Fig. 3, lanes N1, N2, K1, K2); however occasionally, there was some serum nonspecific reactivity to the residual insect cell proteins (evident as differing molecular sizes of proteins) in the semipurified antigen (Fig. 4, lane NC). This reac-

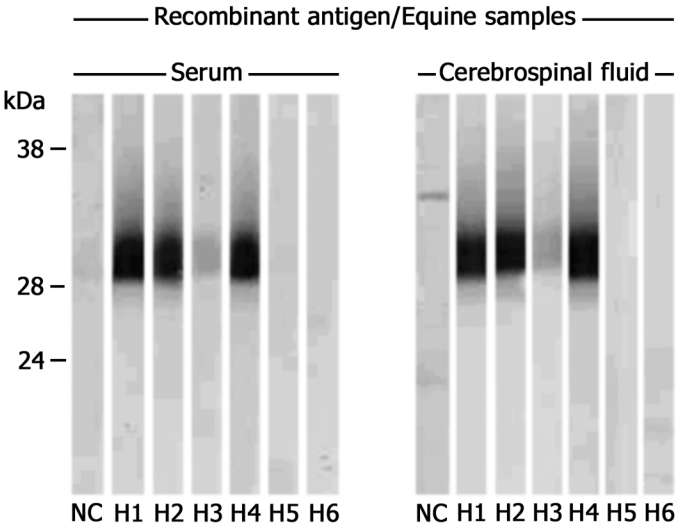

FIGURE 1. Immunoblot of denatured, nonreduced semipurified r-SnSAG1-Bac antigen with serum and cerebrospinal fluid of confirmed EPM horses (lanes H1–H6) and negative control (NC).

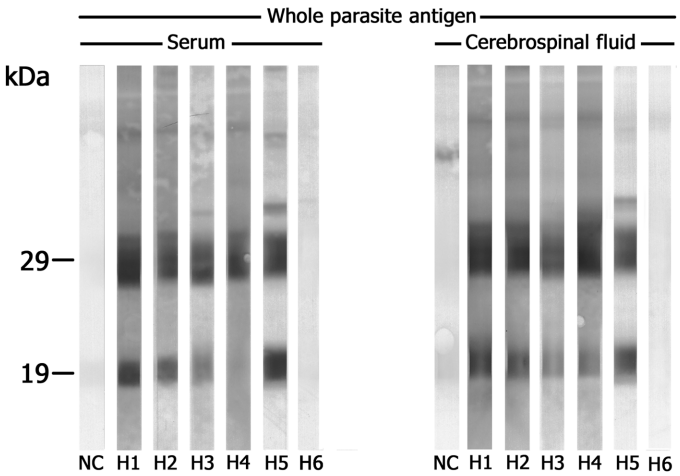

FIGURE 2. Immunoblot of denatured, nonreduced *S. neurona* whole-parasite antigen lysate with serum and cerebrospinal fluid of confirmed EPM horses (lanes H1–H6) and negative control (NC).

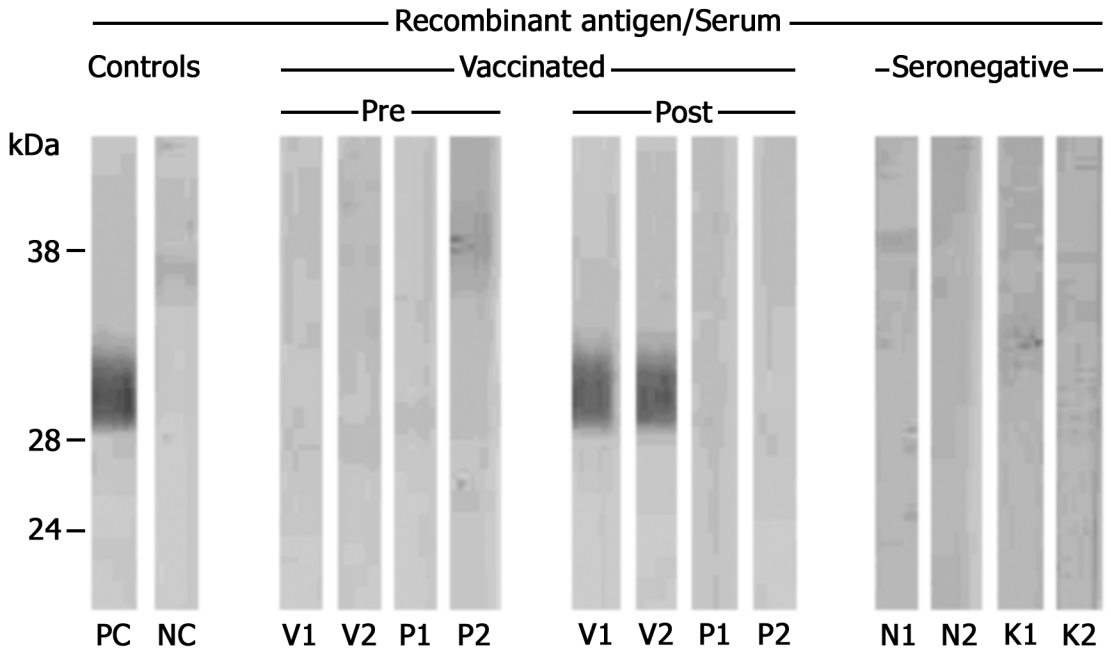

FIGURE 3. Immunoblot of semipurified rSnSAG1-Bac antigen with positive control serum (lane PC), negative control serum (lane NC), and representative sera from pre- and postimmunized horses with *S. neurona* vaccine (lanes V1 and V2), pre- and postinoculated horses with placebo (lanes P1 and P2), and seronegative horses from New Zealand (lanes N1 and N2) and South Korea (lanes K1 and K2).

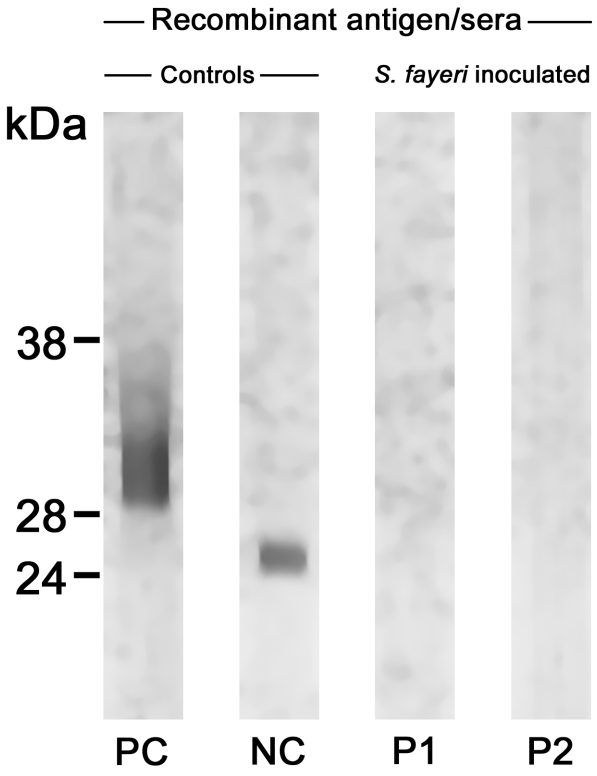

FIGURE 4. Immunoblot of semipurified rSnSAG1-Bac antigen with positive control serum (lane PC), negative control serum, Korean horse (lane NC), and representative sera from *Sarcocystis fayeri* postinoculated ponies (P1 and P2).

tivity was not present when column-purified antigen preparation was used. The 5 sera from the experimentally immunized horses with *S. neurona* vaccine recognized the recombinant SnSAG1-Bac antigen, and no reactivity was seen with the 2 vaccine negative control (placebo-vaccinated group) sera (Fig. 3, lanes V1, V2, P1, P2). The 3 sera from the experimentally inoculated *S. fayeri* ponies showed no reactivity to the SnSAG1-Bac antigen (Fig. 4, lanes P1, P2).

The recombinant SnSAG1-Bac antigen was also well recognized as an ~31-kDa protein by immunoblot analysis with postimmune sera of *S. neurona*–experimentally inoculated nonequid species: skunk, cat, and rabbit (Fig. 5). No reactivity with rSnSAG1-Bac was seen with any of the *S. neurona* preinoculated sera from the skunk, the 4 cats, or the rabbit and the sera from a negative control cat, pre and postinoculated with equine dermal cells. The recombinant SnSAG1-Bac antigen showed no cross-reactivity when probed with sera from experimentally inoculated rabbits (polyclonal *N. caninum*, *N. hughesi*, and *Hammondia* sp. antibodies) but did show reactivity at an ~31-kDa antigen with *S. falcatula* polyclonal antibodies from rabbit (Fig. 6, lane 2). Finally, no cross-reactivity was seen to the recombinant antigen with the use of serum from an experimentally inoculated pony with *T. gondii* oocysts.

DISCUSSION

Current serologic methods, such as immunoblot analysis or IFAT, that use whole culture–derived parasites as antigen are effective in detecting *S. neurona* antibodies, but they are hampered by the difficulty in purifying *S. neurona* merozoites in quantity and in the same stage of replication. The cell surface antigens of protozoans have been the focus of extensive research for their diagnostic potential because they are exposed to the immune system at the initiation of infection (Parmley et

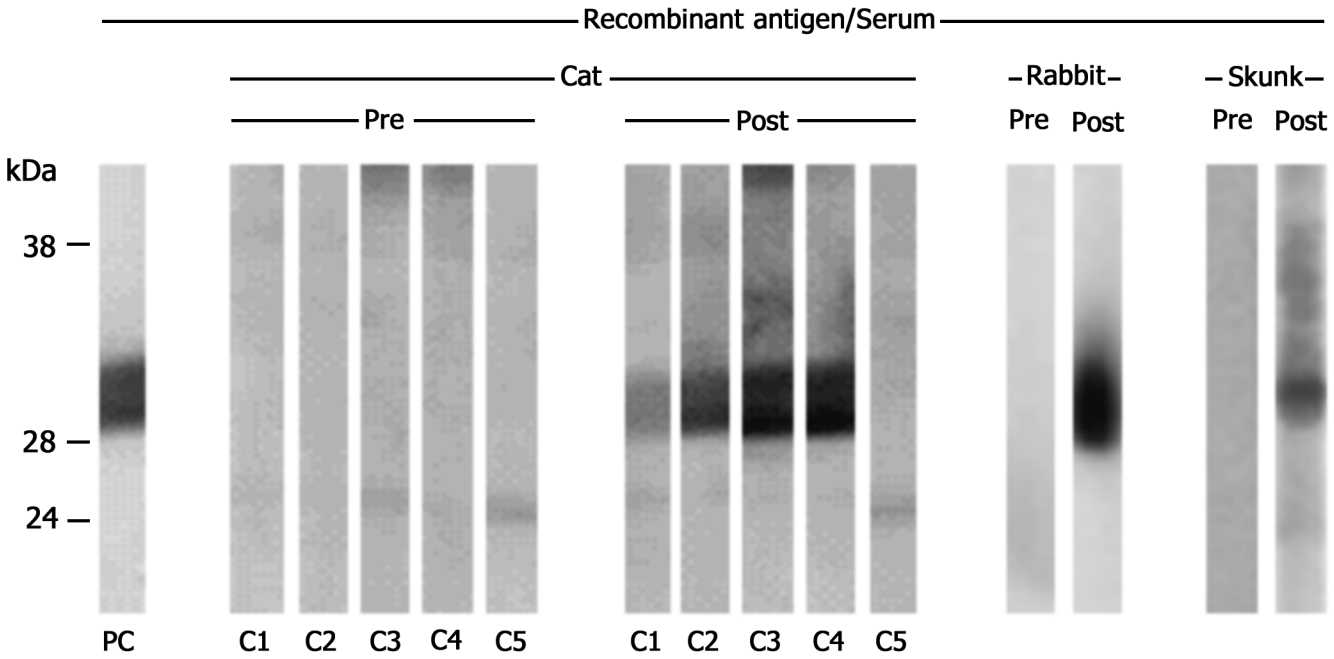

FIGURE 5. Immunoblot of semipurified rSnSAG1-Bac antigen with sera of nonequids, including positive control (lane PC), pre- and postinoculated cats with *S. neurona* merozoites (lanes C1–C4), pre and postinoculated cat with host cells (lane C5), pre- and postinoculated rabbit with *S. neurona* merozoites, and pre- and postinoculated skunk with *S. neurona* sporulated sporocysts.

al., 1992; Harning et al., 1996; Nishikawa et al., 2001). In 2002, the *SnSAG1* gene sequence was reported and characterized as a gene encoding an ~29-kDa immunodominant antigen (Ellison et al., 2002). This gene was chosen for further analysis for its diagnostic potential as a recombinant antigen, as expressed in insect cells via the baculovirus expression system. The rationale behind using this system is that the antigen generated more likely would be similar to the parasite antigen in terms of post-translational modifications because both *S. neurona* and insect cells are eukaryotes. Also, horses would be less likely to have antibodies to any residual insect cell proteins compared with an *Escherichia coli* system used for recombinant protein expression. This system is suitable for large-scale development of antigens that can be used for a wide variety of scientific purposes, such as diagnostics, therapeutics, and structural and functional studies. In our investigation, the recombinant SnSAG1-Bac antigen was successfully expressed and evaluated for its specificity to detect *S. neurona* antibodies by immunoblot analysis. The production of rSnSAG1-Bac antigen in insect cells by the baculovirus system is less time consuming and labor intensive and, above all, proved to be as reliable an antigen as the whole parasite when evaluating only *S. neurona* 29-kDa protein reactivity, compared with the production of whole parasites as an antigen source for current *S. neurona* testing formats.

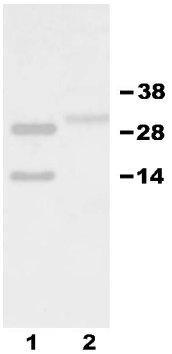

FIGURE 6. Immunoblot of denatured, nonreduced *Sarcocystis falcatula* merozoites (lane 1) and semipurified rSnSAG1-Bac antigen (lane 2) probed with rabbit polyclonal antibodies developed against *S. falcatula* merozoites.

The recombinant SnSAG1-Bac antigen was found to be ~31 kDa in size (in contrast to the ~29-kDa *S. neurona* SAG1 antigen) because the leader peptide of the fusion protein was derived from the baculovirus expression vector. Hence, the size of the resulting recombinant protein is the sum of the SnSAG1 protein and the leader peptide.

The serum and CSF samples of 4 of 6 naturally infected EPM horses reacted with nonreduced recombinant antigen, whereas 5 of 6 reacted with *S. neurona* nonreduced whole-parasite antigen by immunoblot analysis. Initially, this result might seem discouraging; however, several conditions might explain it, such as a relative titer to the specific SAG1, an antigenic difference of SnSAG1 among *S. neurona* isolates infecting those specific horses (Hyun et al., 2003), or the presence of other immunodominant proteins comigrating on 1-dimensional gels with SnSAG1 in whole-parasite lysate. One (H6) of 2 unreactive serum/CSF samples (H5 and H6) to rSnSAG1-Bac, which did react to *S. neurona* whole-parasite antigen, might have reacted with a protein of similar molecular size in the whole-parasite lysate, but not the actual SnSAG1 antigen, which is not discernible by the current 1-dimensional gels in this study. *Toxoplasma gondii* has a number of surface antigens with similar molecular sizes (i.e., SRS2 [39.5 kDa] and SAG5 [39.1 kDa]), which would comigrate on a 1-dimensional gel (Boothroyd et al., 1998). Of the nonreactive EPM-confirmed horses, 1 horse

was documented to have an antigenic variant of *S. neurona* (Hyun et al., 2003). Further studies should focus on the comparison of the *SAG* gene family in various isolates of *S. neurona* to address this issue.

The *S. neurona* seronegative equine population from Jeju Island, South Korea, and New Zealand had no reactivity to the rSnSAG1-Bac antigen. This population came from an area with no reported cases of EPM in horses and in which the definitive host, *Didelphis virginiana*, does not reside. These horses were a variety of ages and pasture fed, suggesting normal exposure to other *Sarcocystis* species. All postimmunization serum samples from horses involved in a *S. neurona* vaccine trial were positive by immunoblot to the ~31-kDa rSnSAG1-Bac antigen, and no antibody reactivity was seen with the preimmunized or placebo horse groups to this antigen. The lack of immunoreactivity to the rSnSAG1-Bac antigen from the sera of *S. fayeri*–inoculated ponies indicates that this antigen, as used in this study, is not recognized by equine *S. fayeri* antibodies.

The results with *S. neurona* postinoculated sera of experimentally inoculated nonequids (the cats, the rabbit, and the skunk) that were positive by immunoblot analysis with recombinant SnSAG1-Bac antigen is quite significant because these species were inoculated with different routes and stages of *S. neurona* parasites. The cats and the rabbit were inoculated by parenteral injection with *S. neurona* merozoites, whereas the skunk was inoculated orally with sporocysts of *S. neurona*. Moreover, 4 of 5 cats (the fifth cat was not inoculated with parasites) were inoculated with 2 different isolates: 2 cats (C1 and C2) with Sn-UCD1 isolate and the other 2 cats (C3 and C4) with Sn-MuCat2 isolate (Fig. 5). Four of these cat sera recognized the recombinant antigen. In addition, sera against *N. caninum*, *N. hughesi*, *Hammondia* spp., and *T. gondii* did not have any cross-reactivity to the recombinant SnSAG1-Bac antigen, although cross-reactivity was seen with antiserum to *S. falcatula* (Fig. 6). However, this result is not surprising considering the close evolutionary relationship of these 2 parasites (Dame et al., 1995; Marsh, Barr et al., 1999) and previously reported cross-reactivity (Marsh et al., 1997). This provides further evidence for the need to compare the *SAG1* gene sequence of both *S. neurona* and *S. falcatula*.

Surface antigen and SAG1-related sequences form a group of related proteins (Boothroyd et al., 1998). Most of these proteins have similar amino acid motifs and conserved cysteine residues, which suggest a similar secondary and tertiary structure. The loss of detectable antibody reactivity after reduction of the recombinant SnSAG1-Bac antigen suggests that conformation plays a role in the antibodies detected and will affect detection of antibodies or immunoreactive proteins when whole *S. neurona* merozoite lysates are used in immunoblot analysis. Alteration of the antibody detection methods to evaluate recognition of reduced antigens was not evaluated extensively in this study; however, such additional studies could reveal specific conformational epitopes that could cause cross-reactivity with *S. falcatula*. This is based on antigenic and DNA sequence comparison studies that use related organisms with characterized SAG1 proteins, such as *N. caninum* and *N. hughesi* (Marsh, Howe et al., 1999), and SAG1 conserved cysteine residues between *S. muris* and *S. neurona* (Ellison et al., 2002). It would be useful to include additional experimentally derived antibodies and antigens of *S. falcatula* to examine differences in cross-reactivity of these related parasites.

This study indicates that the recombinant SnSAG1-Bac antigen can be a potential diagnostic antigen for serodiagnosis of *S. neurona* because it was recognized by sera of naturally infected and experimentally immunized horses and nonequid species. Further studies are required to develop a test based on the enzyme-linked immunosorbent assay for improved quantification of *S. neurona*–specific antibody levels in clinically affected and seropositive-normal horses. Finally, this expression system suggests that other *S. neurona*–specific antigens could potentially be expressed in insect cells, thereby providing additional diagnostic or vaccine candidate proteins.

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LITERATURE CITED

BARR, B. C., J. D. ROWE, K. W. SVERLOW, R. H. BONDURANT, A. A. ARDANS, M. N. OLIVER, AND P. A. CONRAD. 1994. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. Journal of Veterinary Diagnostic Investigation **6:** 207–215.

BERNARD, A., M. PAYTON, AND K. R. RADFORD. 2000. Protein expression in the baculovirus system. *In* Current protocols in protein science, B. M. Dunn, J. E. Coligan, H. L. Ploegh, D. W. Speicher, and P. T. Wingfield (eds.). John Wiley & Sons, Inc., New York, New York, p. 5.5.1–5.5.13.

BOOTHROYD, J. C., A. HEHL, L. J. KNOLL, AND I. D. MANGER. 1998. The surface of *Toxoplasma*: More and less. International Journal for Parasitology **28:** 3–9.

BUTCHER, M., J. LAKRITZ, A. HALANEY, K. BRANSON, G. D. GUPTA, J. KREEGER, AND A. E. MARSH. 2002. Experimental inoculation of domestic cats (*Felis domesticus*) with *Sarcocystis neurona* or *S. neurona*–like merozoites. Veterinary Parasitology **107:** 1–14.

CHEADLE, M. A., C. A. YOWELL, D. C. SELLON, M. HINES, P. E. GINN, A. E. MARSH, R. J. MACKAY, J. B. DAME, AND E. C. GREINER. 2001. The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. International Journal for Parasitology **31:** 843–849. [Erratum in International Journal for Parasitology **31:** 1719.]

———, S. M. TANHAUSER, J. B. DAME, D. C. SELLON, M. HINES, P. E. GINN, R. J. MACKAY, AND E. C. GREINER. 2001. The nine-banded armadillo (*Dasypus novemcinctus*) is an intermediate host for *Sarcocystis neurona*. International Journal for Parasitology **31:** 330–335.

CHEN, G., H. CHEN, H. GUO, AND H. ZHENG. 2002. Protective effect of DNA-mediated immunization with a combination of SAG1 and IL-2 gene adjuvant against infection of *Toxoplasma gondii* in mice. Chinese Medical Journal **115:** 1448–1452.

COHEN, N. D., AND R. J. MACKAY. 1997. Interpreting immunblot testing of cerebrospinal fluid for equine protozoal myeloencephalitis. Compendium on Continuing Education for the Practicing Veterinarian **19:** 1176–1181.

DAME, J. B., R. J. MACKAY, C. A. YOWELL, T. J. CUTLER, A. E. MARSH, AND E. C. GREINER. 1995. *Sarcocystis falcatula* from passerine and psittacine birds: Synonymy with *Sarcocystis neurona*, agent of equine protozoal myeloencephalitis. Journal of Parasitology **81:** 930–935.

DUARTE, P. C., B. M. DAFT, P. A. CONRAD, A. E. PACKHAM, AND I. A. GARDNER. 2003. Comparison of a serum indirect fluorescent antibody test with two western blot tests for the diagnosis of equine protozoal myeloencephalitis. Journal of Veterinary Diagnostic and Investigation **5:** 8–13.

DUBEY, J. P., AND G. DESMONTS. 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vetetrinary Journal **19:** 337–339.

———, M. M. GARNER, M. D. STETTER, A. E. MARSH, AND B. C. BARR. 2001. Acute *Sarcocystis falcatula*–like infection in a carmine bee-eater (*Merops nubicus*) and immunohistochemical cross reactivity between *Sarcocystis falcatula* and *Sarcocystis neurona*. Journal of Parasitology **87:** 824–832.

———, D. S. LINDSAY, W. J. SAVILLE, S. M. REED, D. E. GRANSTROM, AND C. A. SPEER. 2001. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Veterinary Parasitology **95:** 89–131.

———, W. J. SAVILLE, D. S. LINDSAY, R. W. STICH, J. F. STANEK, C. A. SPEER, B. M. ROSENTHAL, C. J. NJOKU, O. C. H. KWOK, S. K. SHEN, AND S. M. REED. 2000. Completion of the life cycle of *Sarcocystis neurona*. Journal of Parasitology **86:** 1276–1280.

———, ———, J. F. STANEK, D. S. LINDSAY, B. M. ROSENTHAL, M. J. OGLESBEE, A. C. ROSYPAL, C. J. NJOKU, R. W. STICH, O. C. KWOK, S. K. SHEN, A. N. HAMIR, AND S. M. REED. 2001. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): Evidence for natural infection with sarcocysts, transmission of infection to opossums (*Didelphis virginiana*), and experimental induction of neurologic disease in raccoons. Veterinary Parasitology **100:** 117–119.

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*. International Journal for Parasitology **32:** 217–225.

FENGER, C. K., D. E. GRANSTROM, A. A. GAJADHAR, N. M. WILLIAMS, S. A. MCCRILLIS, S. STAMPER, J. L. LANGEMEIER, AND J. P. DUBEY. 1997. Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis* sp. sporocysts from the opossum (*Didelphis virginiana*). Veterinary Parasitology **68:** 199–213.

———, ———, J. L. LANGEMEIER, S. STAMPER, J. M. DONAHUE, J. S. PATTERSON, A. A. GAJADHAR, J. V. MARTENIUK, Z. XIAOMIN, AND J. P. DUBEY. 1995. Identification of opossums (*Didelphis virginiana*) as the putative definitive host of *Sarcocystis neurona*. Journal of Parasitology **81:** 916–919.

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. Journal of Veterinary Diagnostic Investigation **5:** 88–90.

GUPTA, G. D., J. LAKRITZ, J. H. KIM, D. Y. KIM, J. K. KIM, AND A. E. MARSH. 2002. Seroprevalence of *Neospora*, *Toxoplasma gondii* and *Sarcocystis neurona* antibodies in horses from Jeju Island, South Korea. Veterinary Parasitology **106:** 193–201.

HARNING, D., J. SPENTER, A. METSIS, J. VUUST, AND E. PETERSEN. 1996. Recombinant *Toxoplasma gondii* surface antigen 1 (P30) expressed in *Escherichia coli* is recognized by human *Toxoplasma*-specific immunoglobulin M (IgM) and IgG antibodies. Clininical and Diagnostic Laboratory Immunology **3:** 355–357.

HAUMONT, M., L. DELHAYE, L. GARCIA, M. JURADO, P. MAZZU, V. DAMINET, V. VERLANT, A. BOLLEN, R. BIEMANS, AND A. JACQUET. 2000. Protective immunity against congenital toxoplasmosis with recombinant SAG1 protein in a guinea pig model. Infection and Immunity **68:** 4948–4953.

HYUN, C., G. D. GUPTA, AND A. E. MARSH. 2003. Sequence comparison of *Sarcocystis neurona* surface antigen from multiple isolates. Veterinary Parasitology **112:** 11–20.

KIMBITA, 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. Veterinary Parasitology **102:** 35–44.

LIANG, F. T., D. E. GRANSTROM, X. M. ZHAO, AND J. F. TIMONEY. 1998. Evidence that surface proteins Sn14 and Sn16 of *Sarcocystis neurona* merozoites are involved in infection and immunity. Infection and Immununity **66:** 1834–1838.

MACKAY, R. J. 1997. Equine protozoal myeloencephalitis. Veterinary Clinics of North American Equine Practice **13:** 79–96.

MARSH, A. E., B. C. BARR, J. MADIGAN, J. LAKRITZ, AND P. A. CONRAD. 1996. Sequence analysis and polymerase chain reaction amplification of small subunit ribosomal DNA from *Sarcocystis neurona*. American Journal of Veterinary Research **57:** 975–981.

———, ———, L. TELL, D. D. BOWMAN, P. A. CONRAD, C. KETCHERSIDE, AND T. GREEN. 1999. Comparison of the internal transcribed spacer, ITS-1, from *Sarcocystis falcatula* isolates and *Sarcocystis neurona*. Journal of Parasitology **85:** 750–757.

———, ———, ———, M. KOSKI, E. GREINER, J. DAME, AND P. A. CONRAD. 1997. In vitro cultivation and experimental inoculation of *Sarcocystis falcatula* and *Sarcocystis neurona* merozoites into budgerigars (*Melopsittacus undulatus*). Journal of Parasitology **83:** 1189–1192.

———, D. K. HOWE, G. WANG, B. C. BARR, N. CANNON, AND P. A. CONRAD. 1999. Differentiation of *Neospora hughesi* from *Neospora caninum* based on their immunodominant surface antigen, SAG1 and SRS2. International Journal for Parasitology **29:** 1575–1582.

———, C. HYUN, B. C. BARR, R. TINDALL, AND J. LAKRITZ. 2002. Characterization of monoclonal antibodies developed against *Sarcocystis neurona*. Parasitology Research **88:** 501–506.

———, 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. Veterinary Parasitology **95:** 143–154.

MAYHEW, I. G., A. DELAHUNTA, R. H. WHITLOCK, L. KROOK, AND J. B. TASKER. 1978. Spinal cord disease in the horse. Cornell Veterinarian **68:** 1–207.

NIELSEN, H. V., S. L. LAUEMOLLER, L. CHRISTIANSEN, S. BUUS, A. FOMSGAARD, AND E. PETERSEN. 1999. Complete protection against lethal *Toxoplasma gondii* infection in mice immunized with a plasmid encoding the SAG1 gene. Infection and Immunity **67:** 6358–6363.

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. Journal of Clinical Microbiology **39:** 3987–3991.

PARMLEY, S. F., G. D. SGARLATO, J. MARK, J. B. PRINCE, AND J. S. REMINGTON. 1992. Expression, characterization, and serologic reactivity of recombinant surface antigen P22 of *Toxoplasma gondii*. Journal of Clinical Microbiology **30:** 1127–1133.

SAVILLE, W. J. A., J. P. DUBEY, M. J. OGLESBEE, C. D. SOFALY, A. E. MARSH, E. ELITSUR, M. C. VIANNA, D. S. LINDSAY, AND S. M. REED. Experimental infection of ponies with *Sarcocystis fayeri* and differentiation from *Sarcocystis neurona* infections in horses. Journal of Parasitology, in press.

ROMAND, S., P. THULLIEZ, AND J. P. DUBEY. 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitology Research **84:** 50–53.

TANHAUSER, S. M., M. A. CHEADLE, E. T. MASSEY, B. A. MAYER, D. E. SCHROEDTER, J. B. DAME, E. C. GREINER, AND R. J. MACKAY. 2001. The nine-banded armadillo (*Dasypus novemcinctus*) is naturally infected with *Sarcocystis neurona*. International Journal for Parasitology **31:** 325–329.

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. Veterinary Parasitology **95:** 273–282.