

QUALITATIVE EVALUATION OF SELECTIVE TESTS FOR DETECTION OF *NEOSPORA HUGHESI* ANTIBODIES IN SERUM AND CEREBROSPINAL FLUID OF EXPERIMENTALLY INFECTED HORSES

Andrea E. Packham, Patricia A. Conrad, W. David Wilson*, Lisa V. Jeanes*, Karen W. Sverlow†, Ian A. Gardner*, Barbara M. Daft‡, Antoinette E. Marsh‡, Byron L. Blagburn§, Gregory L. Ferraro||, and Bradd C. Barr†

Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, California 95616. e-mail: aepackham@ucdavis.edu

ABSTRACT: *Neospora hughesi* is a newly recognized protozoan pathogen in horses that causes a myeloencephalitis similar to *Sarcocystis neurona*. There are no validated serologic tests using the gold standard sera that are currently available to detect specific *N. hughesi* antibodies and, thus, no tests available to detect antemortem exposure or estimate seroprevalence in the horse. The objectives of the present study were to establish a bank of gold standard equine sera through experimental infections with *N. hughesi* and to assess several serologic tests for the detection of related protozoan antibodies. Seven horses were inoculated with *N. hughesi* tachyzoites, and 7 horses received uninfected cell culture material. The horses were monitored, and blood and cerebrospinal fluid were collected repeatedly over a 4-mo period. With the sera, 4 different serologic techniques were evaluated, including a whole-parasite lysate enzyme-linked immunosorbent assay (ELISA), a recombinant protein ELISA, a modified direct agglutination test, and an indirect fluorescent antibody test. Qualitative and quantitative evaluation of the results showed that the *N. hughesi* indirect fluorescent antibody test (IFAT) consistently discriminated between experimentally infected and noninfected horses, using a cutoff of 1:640. Sera from 3 naturally infected horses had titers >1:640. Cerebrospinal fluid in all but 1 infected horse had very low *N. hughesi* IFAT titers (<1:160), starting at postinoculation day 30.

Sporadic cases of neosporosis have been reported in horses in the United States (Dubey and Porterfield, 1990; Gray et al., 1996; Lindsay et al., 1996; Marsh et al., 1996; Daft et al., 1997; Hamir et al., 1998; Cheadle et al., 1999) and France (Pronost et al., 1999). Initially, these were thought to be caused by *Neospora caninum*, but subsequent characterization of the first isolate of this equine *Neospora* organism (Marsh et al., 1996) revealed a unique protozoan that was closely related to, but distinct from, *N. caninum*. This new organism was named *N. hughesi* (Marsh et al., 1998) and was further characterized and shown to be antigenically and molecularly distinct from *N. caninum* (Cheadle et al., 1999; Marsh et al., 1999; Spencer et al., 2000; Walsh et al., 2000; Dubey, Liddell et al., 2001; Walsh et al., 2001). Among the currently reported cases of equine neosporosis, 2 were characterized by abortion (Dubey and Porterfield, 1990; Pronost et al., 1999), 1 by neonatal encephalomyelitis (Lindsay et al., 1996), 1 by visceral neosporosis (Gray et al., 1996), and 4 by adult myeloencephalitis, with clinical signs like those seen with equine protozoal myeloencephalitis (EPM) (Marsh et al., 1996; Daft et al., 1997; Hamir et al., 1998; Cheadle et al., 1999). The 4 cases of EPM-like disease caused by *N. hughesi* infections necessitate the broadening of the clinical definition of EPM to include both *S. neurona* and *N. hughesi* as etiologic agents.

Whereas there are several published reports that indicate a moderate to high seroprevalence of *S. neurona* infection in U.S. horses, similar information regarding the seroprevalence of *N. hughesi* infection in horses is limited and contradictory. Initial

reports have documented a variable percentage of horses in different geographic locations to be seropositive for *Neospora*-reacting antibodies at different titer values (Cheadle et al., 1999; Dubey, Kerber et al., 1999; Dubey, Romand et al., 1999; Dubey, Venturini et al., 1999; Lindsay, 2001; Pitel et al., 2001; Vardeleon et al., 2001). However, the significance of these results is uncertain for several reasons. First, *N. caninum* was the antigen used in the serologic tests for some of these studies, yet characterized organisms of *N. caninum* have not been isolated from horses. Second, no established 'gold standard' sera were used to permit evaluation of accuracy (sensitivity and specificity) in these serologic methods at selected cut-off values. Therefore, it was not possible to distinguish true seropositivity to *Neospora* from the detection of nonspecific cross-reacting antibodies to other related protozoans, particularly *S. neurona*. Typically, gold standard panels consist of a large bank of sera from horses that are confirmed to be exposed or infected with a particular organism. Because there have been only 8 reported cases of equine neosporosis, from which only 3 isolates have been characterized, the establishment of a gold standard serum panel from naturally infected animals is not feasible.

The primary purpose of the present study was to create a bank of gold standard sera by experimentally infecting a group of horses with *N. hughesi* and using their postinoculation sera to qualitatively evaluate 4 different serologic tests, including a whole-parasite lysate enzyme-linked immunosorbent assay (wELISA), a recombinant antigen ELISA (rELISA), a modified direct agglutination test (MAT), and an indirect fluorescent antibody test (IFAT). We sought to determine which tests and which cut-off values could be used to detect specific antibodies reliably in horses and to differentiate between infected and noninfected groups. Secondary objectives were to determine whether there were any significant changes in cerebrospinal fluid (CSF) titers or protein concentrations and to monitor clinical behavior in horses after experimental infection.

MATERIALS AND METHODS

Animals

Fourteen clinically normal, *S. neurona* antibody-negative horses were selected for inclusion in the study from a pool of approximately

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* Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616.

† California Animal Health and Food Safety Laboratory, School of Veterinary Medicine, University of California, Davis, California 95616.

‡ Department of Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211.

§ Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, Alabama 36849.

|| Center for Equine Health, School of Veterinary Medicine, University of California, Davis, California 95616.

40 horses first screened by immunoblotting of serum (Equine Biodiagnostic, Inc. [EBI], Lexington, Kentucky). Horses that tested negative for *S. neurona* antibodies were then subjected to a detailed physical examination, including a complete neurological and lameness evaluation that was recorded on videotape. Horses had no evidence of intestinal parasites based on the absence of parasite eggs in fecal flotation tests and had normal hematologic and biochemical parameters based on complete blood counts and serum biochemistry profiles. The 14 selected horses ranged in age from 5 to 18 yr and included 6 mares and 8 geldings of various breeds (6 Thoroughbreds, 2 Quarterhorses, 1 Hanovarian, 3 Holsteiners, 1 Oldenburg, and 1 Warmblood).

Horses were allocated in 2 groups, an experimental group (nos. 1–7) and a control group (nos. 8–14), each comprising 7 horses. Allocation of the horses was random, with the exception that 2 horses were donated for use with the stipulation that they be assigned to the control group to avoid compromising future studies to which they had been assigned. Clinicians and animal health technicians responsible for clinical monitoring were blinded as to which horses were assigned to each group. All horses were weighed on an electronic scale at the start of the study and at monthly intervals thereafter to permit accurate computation of drug doses.

Horses in the experimental infection group were inoculated intramuscularly (IM) in the pectoral muscle with 5 ml of minimum essential medium (MEM) containing 5×10^7 tachyzoites of the *N. hughesi* isolate (Marsh et al., 1996), grown on equine dermal (ED) cells and prepared as described below. Control horses were inoculated IM with an equal volume of cell culture preparation containing uninfected ED cells in 5 ml MEM.

After inoculation, the horses were monitored clinically, recording temperature, pulse, and respiration at 4-hr intervals for the first day, twice daily for a further 21 days, and then daily for up to 87 days after inoculation. Clinical observations included general appearance, demeanor, appetite, fecal consistency, and gait. Blood was collected on postinoculation days (PID) 0, 3, 7, 10, 14, then weekly for 4 mo, and monthly thereafter through 9 mo after inoculation. Fecal samples were collected once weekly for the first 2 wk. Detailed neurological and gait examinations were performed and recorded on videotape at monthly intervals.

Cerebrospinal fluid was collected monthly on approximately PID 0, 30, 60, and 90 in all horses by centesis of the subarachnoid space at the level of the cisterna magna, using published techniques (Mayhew, 1975). Six 2-ml aliquots of CSF were collected by gravity flow after discarding the first 1–2 ml of CSF.

A second *S. neurona* immunoblot test was performed at PID 28 on all study horses. Four of the experimentally infected horses (nos. 2–5) were euthanized by an intravenous injection of 100 ml of Beuthanasia®-D Special (Schering-Plough Animal Health Corporation, Kenilworth, New Jersey) and necropsied at the end of the study between PID 154 and 279. In addition, horse no. 6 died spontaneously of an intestinal herniation through the epiploic foramen on PID 83 and was necropsied. Before euthanasia, horse nos. 2–5 were immunosuppressed by administration of 30 mg (15 ml) dexamethasone orally once daily for 2 wk. In addition, based on persistent neurological deficits, radiographic examination of the cervical spine in horse no. 3 was performed just before euthanasia. All control horses and the remaining 2 infected horses (nos. 1 and 7) were released from the study and returned to pasture.

Serum from 3 naturally infected horses, EN-1 (Marsh et al., 1996), CA-1 (Daft et al., 1997), and AL-1 (Cheadle et al., 1999), served as positive controls. All 3 horses had histologically confirmed natural *Neospora* species infections, and 2 (EN-1 and AL-1) had cultured isolates recovered from brain tissue that were molecularly characterized to be *N. hughesi* parasites. Horse EN-1 was the source of the isolated parasites used to prepare the inoculum in the present study. In addition, sera from 17 horses with *Sarcocystis neurona* myeloencephalitis, confirmed by postmortem lesions and detection of *S. neurona* parasites by immunohistochemistry (IHC) (Dubey, Garner et al., 2001), were used to assess specificity of the *N. hughesi* IFAT and the MAT. Twelve of these serum samples came from horses that were cited in a previous study (Daft et al., 2000).

Preparation of parasite cell culture inoculum

The *N. hughesi* isolate (EN-1) used in this study was originally isolated from the central nervous system (CNS) tissue of an adult horse

in California with clinical signs of EPM (Marsh et al., 1996). The parasite was maintained as previously described (Marsh et al., 1996) *in vitro* on a stationary monolayer of ED cells. When 80% of the ED cells were infected, tachyzoites were harvested for inoculum preparation as previously described (Conrad et al., 1993). Seven horses received 5×10^7 tachyzoites in 5 ml MEM, and 7 horses received a similar IM injection with uninfected ED cells.

Histology and IHC

Formalin-fixed tissues were trimmed, processed routinely through a series of alcohol and xylene solutions, embedded in paraffin, and used to prepare 3- to 4- μ m-thick, H&E-stained slides for microscopic examination. The tissues processed included liver, kidney, heart, lung, spleen, trachea, adrenal gland, peripheral nerve, lymph node, skeletal muscle, multiple sections of gastrointestinal tract, brain, and spinal cord. Immunohistochemistry, as previously described (Miller et al., 2001) using a polyclonal *N. caninum* and *S. neurona* antisera at an optimal dilution of 1:400 and 1:1,600, respectively, was performed on select sections of brain or spinal cord (or both). Sections were either chosen randomly if no lesions were seen or were chosen owing to the presence of inflammatory or degenerative changes within the individual sections.

Whole-parasite lysate ELISA antigen preparation and testing methods

The wELISA was performed as previously described (Louie et al., 1997), with few modifications. Briefly, plates were coated with soluble antigens from sonicated EN-1 tachyzoites, washed, and blocked before storing at 4 C for no more than 1 wk. Test samples were diluted 1:100. Peroxidase-labeled rabbit anti-horse IgG (Jackson ImmunoResearch, Inc., West Grove, Pennsylvania) was diluted 1:15,000. After the plates were developed, the reaction was stopped, and the optical density (OD) was determined at a wavelength of 405 nm with reference wavelength of 490 nm.

Recombinant ELISA antigen preparation and testing

The rELISA was performed as previously described, with minor modifications (Louie et al., 1997). Recombinant antigen 57, from the bovine *N. caninum* isolate BPA-1, was chosen for use in this test based on immunoblot analysis by standard techniques (Laemmli, 1970; Towbin et al., 1979), showing that it could be detected by *N. hughesi* antibodies in infected horse serum. The plates were coated with the antigen diluted to a final concentration of 1 μ g/ml and later blocked with assay buffer containing 10% dimethyl sulfoxide (DMSO) to reduce background. Serum samples and peroxidase-conjugated rabbit anti-equine IgG (Jackson ImmunoResearch) were diluted 1:100 and 1:15,000, respectively, in DMSO assay buffer. After incubation and washing, the plates were developed, the reaction was stopped, and the OD was determined at a wavelength of 450 nm with a reference of 650 nm. Each sample was run in duplicate on coated and uncoated blank wells.

Modified direct agglutination test antigen preparation and testing methods

The MAT was run as previously described by Packham et al. (1998). Briefly, tachyzoites from the EN-1 isolate were used as the test antigen and prepared as before, except for the use of cold Ca-Mg-free phosphate-buffered saline (PBS) (GIBCO Invitrogen Corp., Rockville, Maryland) for washes. Each serum sample was screened using 2 wells in a "U" bottom microtiter plate (Dynatech Laboratories, Inc., Chantill, Virginia). Final serum dilutions were 1:40 and 1:4,000. Fifty microliters of antigen suspension was added to each well. The positive control well contained serum of the naturally infected horse, EN-1, and the negative control well contained PBS with no serum added. Diffuse opacity across the entire diameter of the well was regarded as a positive agglutination reaction, whereas a negative well exhibited a central discrete opaque dot or button.

Indirect fluorescent antibody test antigen preparation and testing methods

The IFAT was performed as previously described for bovine *N. caninum* (Conrad et al., 1993), using antigen slides (Cell Line Associates, Newfield, New Jersey) that were prepared with tachyzoites of the EN-

1 isolate and an initial serum screening dilution of 1:40. The fluorescein-labeled, affinity-purified antibodies directed against species-specific horse IgG (Jackson ImmunoResearch) were diluted 1:1,000 in PBS and added in 10- μ l aliquots to each well. The endpoint titer was the last serum dilution showing distinct, whole-parasite fluorescence. Slides were read blind by 2 independent readers to verify and compare results. The IFAT was also performed on CSF samples using the same methods, with an initial screening dilution of 1:5. All samples found positive at this screening dilution were then titered out to a final dilution. For purposes of comparison, an IFAT previously described (Conrad et al., 1993) using *N. caninum* as the antigen source was also run on serum from the 3 horses naturally infected with *N. hughesi* and on the single CSF sample available from the naturally infected horse, EN-1.

Statistical analysis

Repeated measures analysis of variance was used to assess the effects of experimental infection (fixed factor) on CSF protein, nucleated cells, red blood cell count, and body temperature over time (within factor). A *P*-value of <0.05 was considered significant. For the IFAT, sensitivity and specificity and exact 95% confidence intervals (CI) were calculated using cut-off titers of 1:320 and 1:640 as positive. Calculations were done on day 28 and at the end of the study (~day 120). Reliability of the IFAT assay using fresh as against frozen serum (*n* = 52) and repeatability of the assay with 2 observers (*n* = 55) was assessed using kappa statistics (Fleiss, 1981).

RESULTS

The weight of the horses at the start of the study ranged from 496 to 665 kg and did not fluctuate significantly during the study. All 7 experimentally infected horses, but none of the controls, showed a mild to moderate elevation in rectal temperature of variable duration, beginning 2–4 days after inoculation. The mean rectal temperature of experimentally infected horses was significantly higher (*P* = 0.02) than that of control horses from the afternoon of PID 4 to the morning of PID 6. Mean body temperatures of experimentally infected horses were 0.3–0.7 C higher on days 4 and 5 (morning and afternoon) and on the morning of day 6 but were not significantly different at other times.

Four experimentally infected horses (nos. 2, 4, 5, and 7) and 1 control horse (no. 12) developed transient swelling at the inoculation site in the left pectoral muscles between PID 4 and 13. Five experimental horses (nos. 1, 3, and 4–6) and 3 control horses (nos. 10, 12, and 13) developed transient edema involving the distal limbs, prepuce, or ventral abdomen, beginning on PID 4 and persisting for 2–11 days and in some instances resolving and recurring intermittently. On PID 4, horse no. 3 developed limb edema and a mild fever (38.7 C) that persisted intermittently for 8 days. On PID 6, this horse developed grade 2/5 ataxia (De LaHunta, 1983) with proprioceptive deficits and weakness involving all 4 limbs. Gait deficits showed minor improvement 60 days after first becoming apparent but were still present 9 mo after inoculation at euthanasia. Cervical radiographs of horse no. 3 just before euthanasia revealed minimal osteoarthritis of the cervical facets from C2–3 through C7-T1, and a small (0.7 cm) free, smoothly marginated osseous body with an adjacent smooth osseous defect at the caudodorsal aspect of C1 vertebral lamina. This osseous body was dorsal to the spinal canal and was suggestive of a previous chip fracture.

There was no significant difference in the mean CSF nucleated cell and erythrocyte counts (*P* = 0.37 and 0.14, respectively) between the infected and control horse groups at any time point throughout the study. These mean values were very low and fluctuated through a range of 0.2–1.5 nucleated cells

and 0.8–7.6 erythrocytes per microliter, respectively. Cerebrospinal fluid mean protein values for the experimentally infected horse group tended to increase slightly after inoculation as compared with the control horse group, but this increased trend was not statistically significant (*P* = 0.29). In the control group mean CSF protein values ranged from a low of 64.0 mg/dl on PID 0 to a high of 79.0 mg/dl on PID 60, whereas in the infected horse group the mean values ranged from a low of 70.8 mg/dl on PID 0 to a high of 86.0 mg/dl on PID 60. Among the infected horse group, the most prominent elevation in CSF protein after inoculation occurred in horse no. 3, which also developed clinical ataxia after inoculation. The preinoculation CSF protein concentration in horse no. 3 was 70 mg/dl. After inoculation this increased to 90 mg/dl on PID 30, 106 mg/dl on PID 60 and 90, and 120 mg/dl at necropsy around 9 mo after inoculation.

Of the 7 noninfected and 7 infected horses that were tested on PID 28 by the commercial *S. neurona* immunoblot (EBI), all were negative, with only 1 “low positive” result in infected horse no. 5. The EBI report stated, “Weak positive Western Blot on serum = Low positive Western Blot reactivity. Animal has been exposed to parasites. A weak positive result is a borderline observation and may be equivocal in the diagnosis of EPM.”

On the basis of a detailed examination of cervical vertebrae, spinal canal, and spinal cord at necropsy, there was no significant gross or microscopic change noted to explain the mild clinical signs reported in horse no. 3. A small number of axon digestion chambers were found in 1 lateral funiculus at spinal cord segment C1 of horse no. 3, but these were not considered significant. Horse no. 4 had mild laminitis, with very slight rotation of the third phalanx in the right forelimb. There was essentially no significant histologic finding in the tissues examined from the 5 experimentally infected horses necropsied. A mild vacuolar hepatopathy was evident in the liver of horse nos. 2–5 that was compatible with a mild steroid-induced hepatopathy secondary to the terminal steroid therapy. In horse nos. 4 and 6, 1–2 mild perivascular lymphocytic infiltrates were found in the brain or spinal cord. In horse no. 2, a single small focus of scarring and gliosis was present in the ventral gray matter of the most caudal sacral spinal cord. Incidental lesions included very mild multifocal renal tubular degeneration or scarring (or both), compatible with small incidental renal infarcts, in horse nos. 4 and 5. Immunohistochemistry on select sections of brain or spinal cord using *N. caninum* and *S. neurona* antisera was negative for parasites.

Results for both the wELISA and the rELISA failed to discriminate between the noninfected and the experimentally infected animal groups (Table I). Comparison of the wELISA OD values at several time intervals after inoculation showed that there was no clear difference between the values of noninfected and infected horses. Initially, promising results were obtained with rELISA when screening sera from select time points in infected and noninfected horses. In particular, control sera had consistently low OD values. However, when this rELISA was run on all serum samples, there was extreme variability in OD values among the 7 infected horses (Table I). For example, at 4 wk after inoculation, when increased titers would be expected to be at, or near, peak values within the infected group, 3 of the 7 experimentally infected horses had rELISA values com-

TABLE I. Comparison of test results on serum from *Neospora hughesi* experimentally infected, control, and naturally infected horses.*

Treatment	Horse	PID	wELISA	rELISA	IFAT	MAT
Infected	1	0	2.52	0.40	<80	+
		28	ND	1.06	5,120	+
		120	3.88	0.51	1,280	+
	2	0	1.69	0.18	80	-
		28	3.92	0.78	1,280	+
		120	ND	0.91	5,120	+
	3	0	2.29	0.21	80	-
		28	3.84	0.75	2,560	-
		120	ND	0.30	2,560	-
	4	0	2.63	0.11	<80	+
		28	3.83	0.26	1,280	+
		120	3.98	0.19	1,280	+
	5	0	2.05	0.12	<80	-
		28	3.89	1.00	20,480	+
		120	3.95	0.32	10,240	+
	6	0	1.94	0.09	160	-
		28	4.00	0.45	10,240	+
		120	NS	NS	NS	NS
	7	0	3.62	0.13	160	+
		28	4.00	0.45	1,280	+
		120	ND	1.20	1,280	+
Control	8	0	1.85	0.24	<80	-
		28	2.03	0.32	80	-
		120	ND	0.24	<80	-
	9	0	2.45	0.27	<80	+
		28	1.53	0.23	80	+
		120	ND	0.28	80	+
	10	0	1.65	0.20	<80	+
		28	1.92	0.22	80	+
		120	1.92	0.25	<80	+
	11	0	1.59	0.12	80	-
		28	1.78	0.18	<80	-
		120	2.32	0.14	320	-
12	0	2.34	0.14	160	+	
	28	1.95	0.13	160	+	
	120	2.14	0.12	320	+	
13	0	3.01	0.07	80	-	
	28	2.30	0.08	<80	-	
	120	ND	0.08	<80	-	
14	0	2.09	0.24	<80	+	
	28	2.30	0.22	80	+	
	120	ND	0.16	80	+	
Natural	AL-1	At nec	ND	0.15	1,280	+
	EN-1	At nec	ND	0.13	1,280	+
	CA-1	At nec	ND	ND	5,120	+

* ND, not done; nec, necropsy; NS, no sample.

parable with the highest OD value for the negative group. Further, the values for sera from 2 (EN-1 and AL-1) of the 3 naturally infected horses also were comparable with the OD values of noninfected horses.

The MAT results are summarized in Table I for serum taken on PID 0, 28, and 120 from experimentally infected and noninfected horses. Among the experimentally infected group of horses, 1 horse was false negative at all time points after inoculation. In addition, preinfection sera from 3 of the 7 experimentally infected horses tested false positive. These sera were tested repeatedly with various lots of parasite antigen and test

modifications, all of which gave the same general results. Among the noninfected horse group, sera from 4 of the 7 horses yielded false-positive results at multiple time intervals. In addition, all 17 sera from horses with confirmed *S. neurona* infections tested false positive with the MAT but were found negative by the IFAT.

A consistent difference in serologic response was detected between *N. hughesi*-infected horses and noninfected horses by using the IFAT (Table I). Evaluation of the IFAT titers for several months after inoculation showed that a rising titer for each individual horse could be detected as early as 3–7 days after inoculation (Table II). Peak antibody titers were detected between PID 28 and 35. The antibody titers within the infected horse group remained elevated above the values of the noninfected control group, with some fluctuations in titer values throughout the entire 120-PID monitoring period. Peak antibody titers of infected horses ranged from 1:2,560 to 1:81,920. Following these peak titers, the individual antibody titers of animals within the infected group decreased slightly but remained above the values for the noninfected group. The lowest titer during this period was 1:640 on PID 105 in horse nos. 1 and 4, with a low titer range of 1:640 to 1:1,280 on PID 105–120. The highest titer detected within the noninfected group of horses during this same period was 1:320. A titer of 1:320 was found in the infected horse group only during the early post-inoculation period of the initial rising titers. The IFAT titers for the sera from the naturally infected horses were 1:1,280 (EN-1 and AL-1) and 1:5,120 (CA-1). By comparison, when the sera from these 3 horses were tested using the *N. caninum* IFAT, the endpoint values decreased to 1:320 (EN-1), 1:160 (AL-1), and 1:640 (CA-1), respectively. Sera from 17 horses with confirmed *S. neurona* infections all had *N. hughesi* IFAT titers of <1:40. Based on these qualitative findings, the most conservative cut-off value for differentiating infected from noninfected horses would be 1:640 (Fig. 1; Table II).

On day 28, the IFAT had a sensitivity of 100% (95% CI = 65.2–100%) and a specificity of 100% (95% CI = 65.2–100%) at a cutoff of 1:320. On day 120, the IFAT still had a sensitivity of 100%, but the specificity was lower (71.4%; 95% CI = 30.3–94.9%). Sensitivity and specificity were both 100% on day 28 and day 120 when the cutoff was increased to 1:640. At both cut-off values the reliability of the assay was high (all kappa values > 0.87), when comparing the use of fresh with that of frozen serum and when assessing interobserver variation. The median titer was significantly higher when samples were tested after being frozen (Wilcoxon signed ranks test, $P = 0.001$). Of the 52 samples that were tested both frozen and fresh, 28 had higher titers when tested frozen, 6 had higher titers when tested fresh, and 18 yielded the same result. In frozen sera from several of the infected horses, titers were only 1–2 dilutions higher ($n = 16$), but in a few instances the titers increased by 3–4 dilutions ($n = 5$). Additionally, in 2 of the uninfected control horses, titers for fresh sera were <1:80, but after freezing, the titer for some samples increased to 1:320.

Using the *N. hughesi* IFAT, CSF titers from all the control horses were negative at a screening dilution of 1:5 at all time points (PID 0, 30, 60, 90). Within the infected horse group, there was no detectable IFAT titer on PID 0. On PID 30, the CSF titers in horse nos. 2 and 7 were negative at 1:5. The remaining 5 horses in the infected group had CSF IFAT titers

TABLE II. Antibody titers to *Neospora hughesi* in horses by indirect fluorescent antibody test throughout the study.*

Treat- ment	Horse	PID 0	PID 7	PID 14	PID 21	PID 28	PID 35	PID 56	PID 77	PID 105	PID 120
Infected	1	<80	160	2,560	5,120	5,120	10,240	1,280	2,560	640	1,280
	2	80	320	640	640	1,280	5,120	1,280	5,120	1,280	5,120
	3	<80	160	2,560	5,120	2,560	2,560	1,280	2,560	1,280	2,560
	4	<80	320	1,280	1,280	1,280	2,560	1,280	1,280	640	1,280
	5	<80	320	10,240	10,240	20,480	81,920	5,120	20,480	1,280	10,240
	6	160	2,560	10,240	2,560	10,240	5,120	2,560	2,560	NS	NS
	7	160	640	2,560	2,560	1,280	2,560	1,280	2,560	2,560	1,280
Control	8	<80	<80	<80	<80	<80	80	80	80	80	<80
	9	<80	80	80	<80	<80	160	80	160	80	80
	10	<80	<80	<80	<80	<80	<80	<80	80	<80	<80
	11	80	<80	160	<80	<80	160	320	320	320	320
	12	160	<80	160	160	160	160	80	80	160	320
	13	80	<80	<80	<80	<80	<80	<80	<80	<80	<80
	14	<80	<80	80	80	<80	<80	<80	<80	<80	<80

* NS, no sample.

ranging from 1:5 to 1:40. On PID 60, the CSF titer from only horse no. 7 remained negative at the 1:5 screening dilution, whereas the titers ranged from 1:10 to 1:80 in the remaining infected horses. On PID 90, the CSF titers for both horse no. 1 and horse no. 7 were negative at 1:5, and the remaining 5 horses had CSF titers from 1:10 to 1:40 (CSF from horse no. 6 was collected on PID 83, when it was found dead). Among the 3 naturally infected horses, CSF was available from only 1 animal (EN-1), and it had a *N. hughesi* CSF IFAT titer of 1:2,560. By comparison, the CSF *N. caninum* IFAT titer for this same horse was 1:160.

DISCUSSION

The primary objective of the present study was to obtain gold standard sera from horses infected with *N. hughesi* so that several serologic methods for detection of *N. hughesi*-specific antibodies in the horse could be evaluated. Of the 4 test methods used, only the IFAT consistently differentiated experimentally infected or naturally infected horses, or both, from noninfected horses. Experimental infection of horses with *N. hughesi* did not result in significant clinical disease. Infected horses maintained a positive IFAT titer for *N. hughesi* 4+ mo after inoculation, suggesting persistent infections, although no parasites

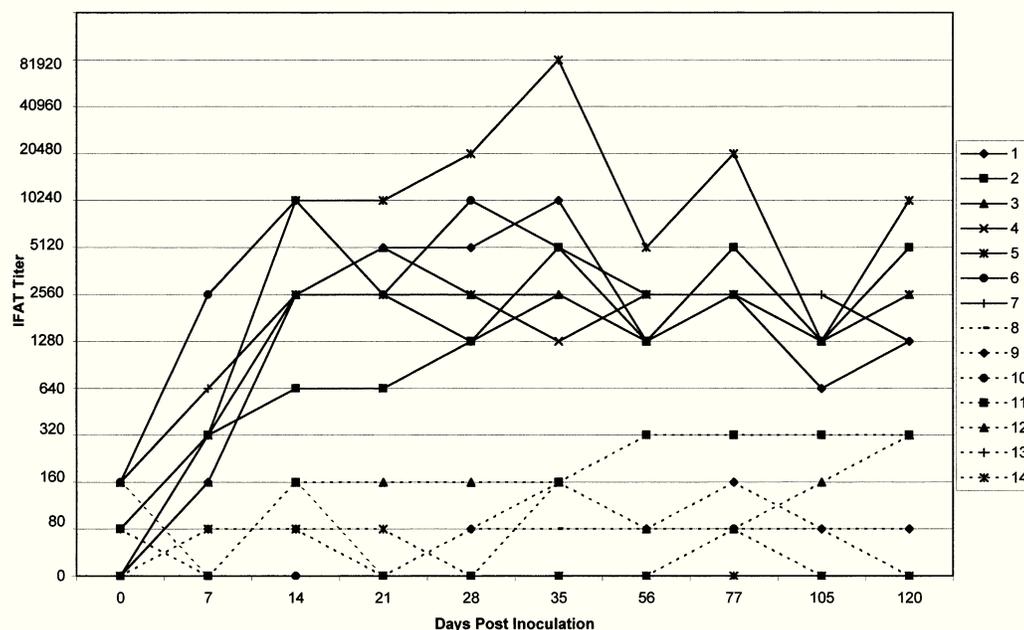


FIGURE 1. Indirect fluorescent antibody test results for horses experimentally infected with *Neospora hughesi* or cell culture control material. Titers of infected horses (nos. 1–7) are represented by solid lines, whereas titers of control horses (nos. 8–14) are represented by dashed lines. Note the clear separation of titer values in the infected and control horses between the suggested cut-off titers of 1:320 and 1:640.

could be detected by either light microscopy or IHC to confirm *N. hughesi* infection.

Unlike previous wELISAs that have been developed and used successfully to detect antibodies directed against specific protozoans (Bjorkman et al., 1994; Pare' et al., 1995; Louie et al., 1997), the wELISA developed for *N. hughesi* could not adequately distinguish between infected and noninfected horses (Table I). Attempts to improve the specificity of this wELISA and reduce the high-background OD values with various blocking methods were unsuccessful. It was anticipated that the recombinant antigen 57, identified initially from *N. caninum* (Louie et al., 1997) but also detected from *N. hughesi*, would solve this background problem. However, in contrast to the wELISA, the rELISA failed to consistently detect specific *Neospora* antibodies in all the infected horses (low sensitivity). Still, problematic high-background values in unexposed horses were not seen with the rELISA. These results indicate that further selection and production of an immunodominant *N. hughesi* protein for use in an rELISA would likely result in a successful and highly specific serologic test. Such an rELISA would have the advantages of being readily available to diagnostic laboratories and would allow for rapid, accurate testing of sera.

The MAT has been shown to be a useful test for quickly screening large numbers of serum samples for reactivity to *N. caninum*, and like the IFAT, the MAT allows the testing of sera from numerous species (Packham et al., 1998; Romand et al., 1998). The greatest disadvantage of this method is the need for continuous cultivation and processing of large quantities of parasites. The test also tends to have a lower specificity, resulting in more false-positive results (Packham et al., 1998). Although the MAT has performed well in the past, when testing sera of other species using the *N. caninum* parasites, in the present study the MAT was extremely problematic because of spontaneous agglutination of the parasites exposed to horse sera, using either *N. caninum* (data not shown) or *N. hughesi* as the antigen source. It seems plausible that the same factors responsible for the high OD values obtained with control sera by using the wELISA may also be responsible for the spontaneous agglutination occurring with the MAT. Further, because we have not encountered this problem of spontaneous agglutination with sera from other species, horse serum might have some unique property responsible for the false agglutination in the MAT and the high OD values with the wELISA. Because of this inherent problem, strict quality control procedures are necessary whenever an MAT is used to evaluate equine sera for *N. hughesi*-specific antibodies.

The IFAT was the only test that consistently and accurately differentiated infected from noninfected horses (see Tables I, II; Fig. 1). Elevated titers were first noted between 3 and 10 days, with peak titers occurring between 35 and 56 days. The values tended to decline and then plateau or to slowly decline over several months. The persistence of relatively high titer values during the entire 4-mo period indicates that the inoculated horses may have been infected persistently. These persistent titers were evident even though parasites or significant inflammatory lesions indicative of a protozoan infection (or both) could not be found. Such a persistent subclinical infection in the absence of detectable parasites or protozoal-linked lesions would be consistent with the persistent infections caused by *N. caninum*

in cattle (Barr et al., 1993; Ho et al., 1996, 1997; Anderson et al., 1997). The pattern with peak antibody titers around 30 PID, followed by a brief decline and plateau of the antibody titers over an extended period, is also similar to that found in cattle with *N. caninum* infections (Conrad et al., 1993).

For a small sample size, determination of a cut-off value for any serologic test is problematic. However, the consistent titer values obtained in this study by using the IFAT method indicate a distinct discrimination between infected and noninfected horses somewhere between antibody titers of 1:320 and 1:640. This titer cut-off range is very similar to that found in our laboratory for cattle (Conrad et al., 1993) and dogs (data not shown). Both qualitative and statistical evaluation support a cut-off value of 1:640. With a 1:640 cut-off value, all noninfected horses in the present study would be considered negative, whereas all the inoculated horses and the sera available from the 3 naturally infected horses would be positive. Further, elevation of titers in frozen, thawed samples would argue for the higher cut-off value of 1:640. Statistical analysis using samples on PID 28 and 120 also indicate that a 1:640 cut-off value would have yielded a sensitivity and specificity of 100%, compared with 100% sensitivity and 71.4% specificity with a 1:320 cut-off value. It may be necessary to adjust this IFAT cut-off value in the future as the pool size of gold standard sera from confirmed natural equine *Neospora* cases expands.

When the serum titers from the 3 naturally *N. hughesi*-infected horses were compared using both the *N. hughesi* and the *N. caninum* (Conrad et al., 1993) IFATs, the *N. caninum* IFAT produced consistently lower titers (1:640, 1:320, 1:160). Two of these naturally infected horses would have been considered negative based on a cut-off value of 1:640. Similar results were obtained when comparing the single CSF fluid obtained from 1 of these naturally infected horses. This comparison of sera and CSF indicates that *N. hughesi* antigen should be used when seroprevalence studies for this parasite are conducted.

Apart from horse no. 3 the only clinical findings noted after experimental *N. hughesi* infection were a transient fever on PID 3–6, some local inflammation or edema at the injection site, and mild limb edema in some horses. The cause of the mild limb dysfunction in horse no. 3 is uncertain because there was no histologic evidence of myeloencephalitis. The total CSF protein levels in this horse were consistently higher than in the remaining infected horses. Radiographs of horse no. 3 before euthanasia revealed a small bone chip, suggesting possible previous trauma, at the C2–3 vertebrae. Perhaps the clinical signs resulted from nonspecific inflammation or edema at this C2–3 site after inoculation, which could also explain the higher CSF protein levels in this horse. Such increased vascular permeability at the blood–brain barrier, allowing leakage of serum proteins and antibodies due to trauma, might help explain why horses diagnosed with EPM based on CSF titers respond so well to treatment but are later found to have no evidence of EPM at necropsy. It is plausible that some of these diagnoses based on CFS titers are the result of trauma and that the clinical signs diminish later with treatment only because the lesions are given enough time to heal.

Although low *N. hughesi* IFAT titers (1:5 to 1:80) were detected in the CSF of all but 1 infected horse after infection, there was no detectable lesion or parasite found within the CNS of the experimentally infected horses. In contrast to these low

N. hughesi CSF titers, the *N. hughesi* CSF titer in the single naturally infected horse was very high (1:2,560). Serum contamination of these CSF samples in the infected group was unlikely, based on both the very low red cell counts in the CSF taps (Miller et al., 1999) and the lack of any statistical difference between the CSF protein concentrations in the infected and the control groups. Therefore, the combined results would suggest that *N. hughesi* CSF titers from horses with undiagnosed CNS disease must be interpreted very cautiously. Low CSF titers may indicate infection but not *N. hughesi*-induced EPM disease, whereas higher CSF titers may be more indicative of clinical *N. hughesi*-induced EPM. As more CSF titers are run from confirmed cases of *N. hughesi*-induced EPM, a cut-off value may emerge that could serve as a more accurate antemortem test for discriminating infection from disease. Current interpretation of an EBI-positive Western blot on CSF states that antibodies to *S. neurona* are detected and that active disease is probable (EBI). However, our finding of low positive *N. hughesi* IFAT titers in the CSF of latently infected horses suggests that similar results might occur after *S. neurona* infection in horses. Because the current immunoblot methodology does not discriminate between low and high antibody concentrations, it seems reasonable that a positive immunoblot result on CSF in horses also would not be able to discriminate between latent infection and EPM disease. The results, therefore, also indicate that a more discriminating method is needed if CSF fluid is to be used for accurate antemortem diagnosis of *S. neurona*-induced EPM. The source of the *N. hughesi* antibodies in CSF after experimental infection is unclear. They might possibly result from some nonspecific vascular inflammation and protein leakage or may actually reflect a slight CNS *N. hughesi* infection below the limits of detection by conventional light microscopy and IHC.

Results for the EBI *S. neurona* immunoblot on PID 28 indicate a lack of nonspecific *S. neurona* cross-reactivity detected in *N. hughesi* seropositive horses. Only horse no. 5 had a borderline low positive result on the *S. neurona* immunoblot after *N. hughesi* infection. Daft et al. (2000) have found that there is no good correlation between *S. neurona* infection and a low positive result on CSF. Apart from this 1 low positive result, the remaining samples from the infected horses at peak titer intervals were negative, indicating that this commercial *S. neurona* Western blot does not readily detect antibodies to *N. hughesi*-infected horses. We did not compare these immunoblot results with a second commercial *S. neurona* Western blot test that lists "nonspecific reactivity" as possibly reflecting a cross-reaction to other protozoa including *Neospora* organisms (Neogen Corp. EPM Testing Lab., Lexington, Kentucky). Our findings at least suggest that caution is indicated to avoid overinterpretation of this nonspecific reactivity result. In addition, sera from 17 horses with *S. neurona* infections confirmed by histology and IHC at necropsy were tested, and the titers from all these horses by IFAT were <1:40, arguing against false-positive cross-reactions with *S. neurona*-specific antibodies.

Sera from horses with confirmed infections of other protozoa, such as *T. gondii*, *N. caninum*, *Eimeria* species, and other *Sarcocystis* species of horses, were not available to assess for possible cross-reacting antibody reactions using this *N. hughesi* IFAT. Our evaluation of the *N. caninum* IFAT would suggest that cross-reacting antibody titers to *N. caninum*-exposed horses

likely would be detected by this *N. hughesi* IFAT but at lower dilutions than would be seen using *N. caninum* antigen slides. Available data from previous reported studies in other mammalian species would suggest that *T. gondii* cross-reacting antibodies also would be detected with the *N. hughesi* IFAT, but the strongest reactions would manifest as apical fluorescence of parasites. This phenomenon is thought to occur because of cross-reactions of antibodies to conserved epitopes within the apical complex of the tachyzoites. Apical fluorescence is focal in nature and is considered to be a negative result. Based on previous comparisons of *N. caninum* IFAT with *T. gondii* IFAT, and other serologic test comparisons between antibodies to these 2 parasites, true positive reactions from cross-reacting *T. gondii* antibodies would be anticipated only at very low titers, most of which would be below the 1:640 cut-off value established in this study (Conrad et al., 1993; Trees et al., 1994; Pare' et al., 1995; Dubey et al., 1996). Seroprevalence studies also suggest that there is a low prevalence of *T. gondii*-positive serology in equine populations (Dubey and Beattie, 1988).

Based on the results of the present study, the IFAT using *N. hughesi* antigen is the only serologic test among those examined that would allow for an accurate assessment of *N. hughesi* exposure and seroprevalence within the equine population. Nonetheless, further investigation of immunodominant *N. hughesi* recombinant proteins in an rELISA should be undertaken to produce a more highly specific and sensitive second-generation *N. hughesi* serology test that would be amenable to a more diverse group of diagnostic laboratories.

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