Evaluation and Comparison of an Indirect Fluorescent Antibody Test for Detection of Antibodies to Sarcocystis neurona, Using Serum and Cerebrospinal Fluid of Naturally and Experimentally Infected, and Vaccinated Horses

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EVALUATION AND COMPARISON OF AN INDIRECT FLUORESCENT ANTIBODY TEST FOR DETECTION OF ANTIBODIES TO SARCOCYSTIS NEURONA, USING SERUM AND CEREBROSPINAL FLUID OF NATURALLY AND EXPERIMENTALLY INFECTED, AND VACCINATED HORSES

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ABSTRACT: The objectives of this study were to evaluate the accuracy of the indirect fluorescent antibody test (IFAT) using serum and cerebrospinal fluid (CSF) of horses naturally and experimentally infected with Sarcozystis neurona, to assess the correlation between serum and CSF titers, and to determine the effect of S. neurona vaccination on the diagnosis of infection. Using receiver-operating characteristic analysis, the areas under the curve for the IFAT were 0.97 (serum) and 0.99 (CSF). Sensitivity and specificity were 83.3 and 96.9% (serum, cutoff 80) and 100 and 99% (CSF, cutoff 5), respectively. Titer-specific likelihood ratios (LRs) ranged from 0.03 to 187.8 for titers between <10 and 640. Median time to conversion was 22–26 days postinfection (DPI) (serum) and 30 DPI (CSF). The correlation between serum and CSF titers was moderately strong (r = 0.6) at 30 DPI. Percentage of vaccinated antibody-positive horses ranged from 0 to 95% between 0 and 112 days after the second vaccination. Thus, the IFAT was reliable and accurate using serum and CSF. Use of LRs potentially improves clinical decision making. Correlation between serum and CSF titers affects the joint accuracy of the IFAT; therefore, the ratio of serum to CSF titers has potential diagnostic value. The S. neurona vaccine could possibly interfere with equine protozoal myeloencephalitis diagnosis.

Sarcozystis neurona is the protozoan parasite most commonly associated with equine protozoal myeloencephalitis (EPM), a common neurological disease of horses in North America (Nappert et al., 1989; Hamir et al., 1992; Daft et al., 2002). The difficulties in establishing a definitive diagnosis of EPM in horses with neurological signs and the limitations of the current diagnostic methods for the disease have been reviewed recently (Furr et al., 2002). The Western blot (WB), and more recently a modified WB test (mWB), have been the ancillary diagnostic tests used in the clinical diagnosis of EPM caused by S. neurona and for serosurveys for antibody against the parasite (Saville et al., 1997; Rossano et al., 2001; Furr et al., 2002). The WB test has also been used for confirmatory purposes in the detection of S. neurona antibodies in the cerebrospinal fluid (CSF) of seropositive horses with clinical signs consistent with EPM (Furr et al., 2002). The high proportion of serum immunoblot–positive horses without signs of EPM has indicated low specificity of these tests in relation to the presence of S. neurona in the central nervous system (CNS) (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Tillotson et al., 1999; Rossano et al., 2001). In addition, a recent study estimated the specificity of the WB using serum and CSF of neurological horses to be 38 and 44%, respectively (Daft et al., 2002).

Ideally, diagnostic tests for EPM should be accurate, quantitative and reliable (reproducible), yield quick results, and inexpensive. Recently, an indirect fluorescent antibody test (IFAT) was compared with the WB and mWB tests for serologic diagnosis of EPM (Duarte et al., 2003). The IFAT was demonstrated to be more accurate than both immunoblots using serum, and the advantage of the quantitative nature of IFAT results, its potential lower cost, and ease of performance were highlighted (Duarte et al., 2003). However, the accuracy of the IFAT in CSF has not been assessed. In addition, the relationship between S. neurona serum and CSF antibody titers and its potential implication in the diagnosis of EPM has not been investigated.

Since 2000, an inactivated S. neurona merozoite vaccine has been conditionally licensed in the United States as an aid in the prevention of EPM caused by exposure to S. neurona. The vaccine was reported to induce antibody production in horses. However, vaccination might potentially complicate interpretation of serum and CSF antibody test results in horses tested for EPM.

The objectives of the present study were to evaluate and compare the accuracy of the IFAT using serum and CSF of horses naturally and experimentally infected with S. neurona, to assess the correlation between serum and CSF antibody titers, and to determine the effect of vaccination on the accuracy of the IFAT for the diagnosis of S. neurona infection.

MATERIALS AND METHODS

Panel of samples

Naturally infected horses: Samples from horses previously enrolled in a validation study of the WB test were obtained (Daft et al., 2002). These samples included serum samples previously used in a comparison of the IFAT and 2 different WB tests (Duarte et al., 2003). Horses with and without neurological disease submitted for necropsy to the California Animal Health and Food Safety Laboratory System between 1996...
and 1999 were enrolled in the study (Daft et al., 2002). Samples of brain and spinal cord were collected and processed for histologic examination. If microscopic lesions consistent with EPM were found, immunostaining for S. neurona was performed (Daft et al., 2002). Horses with histologic lesions compatible with S. neurona infection and parasites demonstrated in lesions by immunostaining (Barr et al., 1994; Dubey et al., 2001) were classified as EPM cases (EPM+). Horses having histologic lesions compatible with S. neurona infection but no parasite demonstrated in lesions were classified as EPM suspects. Horses having no histologic lesion, or inconsistent or insufficient histologic lesions in the CNS for consideration as EPM cases or suspects, or with protozoan parasites other than Sarcocystis spp in the CNS, were classified as negative for EPM (EPM−).

Filtered pens (vaccinated and nonvaccinated horses were housed together), and fed heat-processed (autoclaved at 95 C for 30 min) hay and commercial pelleted feed. Horses were crossbred males and females, aged between 9 mo and 2 yr, and were located at the large animal facilities at FDAH Laboratories, Iowa.

The vaccination protocol consisted of two 1-mL doses of S. neurona vaccine administered intramuscularly 3 wk apart. The vaccine contained a standardized number of formalin-inactivated S. neurona merozoites and adjuvant (Metasim®). Serum and CSF (cisterna magna) samples were collected before the first vaccination (0 DPV1) and then at approximately 14, 28, 112, 240, and 365 days after the second vaccination (DPV2). Samples from 0 DPV1 and from 14, 28, and 112 DPV2 were tested by IFAT at the laboratory of 1 of the authors (P.A.C.).

**Indirect fluorescent antibody test**

The IFAT was performed as previously described (Duarte et al., 2003), using S. neurona merozoites (UCD-1 isolate) as the test antigen (Marsh et al., 1996). Parasites were maintained in vitro (MA104 cells), harvested by scraping, filtered to minimize cell debris (PD-10 Sephadex column, Pharmacia Biotech, Uppsala, Sweden), added to 12-well slides (Cell Line Associates, Newfield, New Jersey) in 10-μl aliquots per well, and air dried. Subsequently, antigen slides were fixed in 10% formalin for 10 min, double washed in phosphate-buffered saline (PBS), air dried again, and stored at −70°C for future use. A monoclonal anti-S. neurona antibody directed against horse-specific IgG (Jackson ImmunoResearch Inc., West Grove, Pennsylvania) and labeled with fluorescein were diluted 1: 1,000 in PBS and added to each well in 10-μl aliquots.

Serum and CSF samples were screened at 1:10 and 1:5 dilutions, respectively. The lower screening dilution was used for CSF because preliminary studies had indicated that IFAT titers in CSF were 2- to 4-fold lower than in serum. If distinct whole-parasite fluorescence was observed at the screening dilution (Conrad et al., 1993), samples were further diluted to obtain end-point titers. The end-point titer was the last serum or CSF dilution showing distinct, whole-parasite fluorescence (Conrad et al., 1993). An arbitrary cutoff titer for a positive test result of 80 for serum and 5 for CSF, horses were classified as having a titer of <10 or <5, respectively. Each slide was read by 1 of the authors (A.E.P.), experienced with the technique, and by a laboratory assistant. All IFAT results were assessed without the reader’s knowledge of the true infection or vaccination status of the horse being tested.

A subset of 67 sera from naturally infected horses was selected for IFAT reliability evaluation. Samples included EPM+ (n = 12), EPM suspects (n = 7), and EPM− (n = 48) horses. The IFAT was run twice on the same aliquot and once on a second aliquot that had been frozen again and thawed fewer times than the first aliquot. Test results were read by 2 readers with different experience with the technique.

Serum samples from the experimentally infected horses from trial 2 at 0 and 84 days postinfection (DPI) were used to assess possible cross-reactivity with other Apicomplexan parasites. Samples were screened by IFAT at 1:10 dilution on antigen slides of Toxoplasma gondii, Neospora caninum, and N. hughesi tachyzoites as previously described (Conrad et al., 1993; Miller et al., 2002; Packham et al., 2002).

**Statistical analysis**

Receiver-operating characteristic analysis was used to compare the areas under the curve (AUCs) for the IFAT using serum and CSF (Greiner et al., 2000). LRs were estimated for each specific titer value using logistic regression analysis as previously described (Sime et al., 1993; Dujardin et al., 1994). Sensitivities and specificities of the IFAT using serum and CSF and exact binomial 95% confidence intervals (CI) were calculated using standard formulas (Thrusfield, 1997). Test results for EPM-suspect horses were presented separately. In the present study an arbitrary cutoff titer for a positive test result of 80 for serum and 5 for CSF was used when dichotomization of IFAT results was necessary for data analysis.

Kappa statistic was calculated as a measure of interobserver, intraobserver, and interaliquot agreement beyond chance for IFAT results (Landis and Koch, 1977). The intraobserver agreement was calculated for each and every experienced reader. The interobserver agreement was calculated using the test results from both readers for the first aliquot. The interaliquot agreement was calculated using a randomly chosen test result from the 2 readings on the first aliquot made by the most experi-
TABLE I. Frequency distribution of *Sarcocystis neurona* serum and CSF IFAT titers for EPM+ and EPM− horses and titer-specific LRs for serum estimated by logistic regression.

<table>
<thead>
<tr>
<th>Titer</th>
<th>EPM+</th>
<th>EPM−</th>
<th>LR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5</td>
<td>0</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>85</td>
<td>14</td>
<td>0.03 (0.0-0.2)</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>6</td>
<td>0.7 (0.0-0.8)</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>1</td>
<td>1.7 (1.3-2.2)</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>1</td>
<td>4.4 (2.2-8.7)</td>
</tr>
<tr>
<td>80</td>
<td>4</td>
<td>0</td>
<td>11.2 (3.7-34.2)</td>
</tr>
<tr>
<td>160</td>
<td>3</td>
<td>2</td>
<td>28.7 (6.1-135.3)</td>
</tr>
<tr>
<td>320</td>
<td>2</td>
<td>1</td>
<td>73.4 (10.1-535.1)</td>
</tr>
<tr>
<td>640</td>
<td>1</td>
<td>0</td>
<td>187.8 (16.7-2,115.6)</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>

**RESULTS**

**Naturally infected horses**

The frequency distributions of serum and CSF IFAT titers for the EPM+ and EPM− horses and the titer-specific LRs for serum are presented in Table I. The LR for a CSF titer of 5 was 29.1 (95% CI = 3.3–254.2). The LR for a CSF titer <5 was 0.0001. No other LR value was estimated for CSF because of the small sample sizes for other titer values.

The AUCs for the IFAT using serum and CSF were 0.97 (95% CI = 0.92–0.995) and 0.999 (95% CI = 0.96–1), respectively. There was no significant difference between these AUCs ($P = 0.082$). The specificity and sensitivity of the IFAT using serum were 83.3% (95% CI = 53.6–97.9%) and 96.9% (95% CI = 91.2–99.4%), respectively. Using CSF, the sensitivity and specificity of the test were 100% (95% CI = 69.2–100%) and 99% (95% CI = 94.4–99.7%), respectively.

The correlation coefficient between serum and CSF titers for the 110 available pairs was 0.43 ($P < 0.01$). The ratio of serum to CSF IFAT titers among EPM+ horses ranged between 2 and 16 (n = 8).

The range of serum titers for the 8 available EPM-suspect horses was between <10 and 160. All 8 suspect horses had CSF titers of <5. Nine of the 100 EPM− horses had non-*S. neurona* protozoan encephalitis. Serum titers for these horses ranged from <10 to 10 (n = 7). All CSF samples (n = 8) had titer values <5. These parasites were not further identified.

Kendall's tau correlation coefficients between serum and CSF titers were estimated for naturally infected, experimentally infected, and vaccinated horses. The correlation coefficient between serum and CSF titers ranged between 16 and 128.

**Experimentally infected horses**

**Trial 1:** Serum and CSF titers between 0 and 30 DPI for the 20 *S. neurona*–infected horses are presented in Table II. All control horses had serum and CSF titers of <5. The sensitivity of the IFAT increased progressively with time, and at approximately 30 DPI, the sensitivity of the test using serum was 75% (95% CI = 51–91%). At the same time, the sensitivity of the test in CSF was 40% (95% CI = 19–64%).

The median time to seroconversion for *S. neurona*–infected horses was 26 days. Time to seroconversion decreased as sporocyst dose increased ($P < 0.01$).

The correlation coefficient between serum and CSF titers at approximately 30 DPI was 0.62 ($P = 0.003$). The ratio of IFAT titers in serum to CSF titers ranged between 16 and 128.

**Trial 2:** Serum and CSF antibody titers for the 8 *S. neurona*–infected horses.
infected horses between 0 and 98 DPI are presented in Table III. One of the 2 control horses had serum and CSF IFAT titers ≤10 and <5, respectively, throughout the study. The serum (and CSF) titer for the second control horse ranged between 20 and 80 (<5–20) during the follow-up period.

The medium times to serum and CSF conversion for the 8 infected horses (nonimmunosuppressed and immunosuppressed) were 22 (range, 9–38) and 30 (range, 22–30) days, respectively. Time to serum and CSF conversion did not significantly differ for the infected nonimmunosuppressed and infected immunosuppressed groups separately (P > 0.05).

The sensitivity of the IFAT using serum and CSF increased progressively with time. The sensitivities of the test at 34 DPI were 75% (95% CI = 35–97%) and 88% (95% CI = 47–99.7%) for serum and CSF, respectively. The sensitivity of the IFAT was 100% from 56 DPI for serum and from 70 DPI for CSF to the end of the study.

The correlation coefficients between serum and CSF titers ranged between 0.53 and 0.84 between 34 and 98 DPI, respectively (all P < 0.05). The correlation coefficients at 26 and 41 DPI were 0.33 and 0.13, respectively (P > 0.05). The ratio of serum titers to CSF titers at 14 DPI2 (n = 11) ranged between 0.5 and 2,040. Ninety-one percent of the ratios were ≤128. At 28 DPI2 (n = 6), the ratio of serum to CSF titers ranged between 32 and 1,024. Sixty-seven percent of the ratios were ≥256.

The median serum to CSF ratio at 28 DPI2 was significantly higher than the median serum to CSF ratio of the naturally infected horses and the experimentally infected horses at comparable times (trial 1 at 30 DPI, trial 2 at 34 DPI) (all P < 0.03).

**DISCUSSION**

In a previous report using a subset of the samples from the naturally infected horses included in the present study, we showed that the IFAT was more accurate than 2 different WB assays for serologic diagnosis of EPM caused by *S. neurona* (Duarte et al., 2003). In the present study the IFAT was shown to be at least as accurate in CSF as in serum for the diagnosis of the disease. Additionally, the IFAT was demonstrated to be reliable (Kappa > 0.7) among readings, readers, and aliquots (Landis and Koch, 1977).

In this study serum and CSF samples from the naturally infected horses were a subset of the horses used in a study by 1 of the authors (Daft et al., 2002). This subset of samples was obtained based on availability, and the titer distribution among these horses might not be representative of the titer distribution in the original set of samples or in the population. A previous study indicated that treatment for EPM might potentially decrease antibody concentration in serum and CSF of naturally infected horses (Furr et al., 2001). Treatment for EPM could also potentially lead to misclassification of a horse’s *S. neurona*
infection status. In this study, 10 horses had a history of treatment with antiprotozoan drugs for EPM. The range of serum and CSF titers for the 3 treated EPM+ horses was comparable with those of nontreated horses (serum, 80–640; CSF, 20–160). In addition, the serum and CSF titers for the 5 EPM– and the 2 EPM-suspect horses treated for EPM were ≤10 and ≤5, respectively. The potential effect of treatment on clearance of S. neurona infection or development of EPM in these horses is unknown.

Ideally, cross-reactivity assessment should be performed using sera of horses infected with other Apicomplexan parasites on S. neurona IFAT slides because that is the actual test situation where cross-reaction might represent a problem. Serum samples from horses previously enrolled in a N. hughesi experimental infection study (Packham et al., 2002) were tested and had S. neurona IFAT titers of <40 at various times post-infection (data not shown). In this study, there were no sera from horses experimentally infected with other Sarcocystis spp. available for evaluation. All horses from Trial 2 had S. neurona titers ≤1:40 at 0 DPI even though mature sarcocysts, morphologically compatible with S. fayeri were evident in skeletal muscles, esophagus, and tongue by the time of necropsy at 98 DPI (Cutler et al., 2001). However, experimental infections with S. fayeri indicate that acute infections might cause false-positive reactions on the S. neurona IFAT (W. J. Saville, pers. comm.). In this study, all S. neurona experimentally-infected horses from Trial 2 tested <80 for T. gondii, N. caninum, and N. hughesi, at the peak of antibody response (84 DPI). In addition, the overall frequency of false-positive reactions in serum of horses without EPM at a cutoff of 1:80 was low (3%). These results corroborate previous reports involving different species, including horses, that cross-reactivity between S. neurona, Sarcocystis spp., T. gondii, and Neospora spp. (Dubey et al., 1996; Dubey, Kerber et al., 1999; Vardeleon et al., 2001; Packham et al., 2002) is minimal.

Findings from both experimental infection trials showed that IFAT accuracy varied with time after infection and with the cutoff chosen, but at 30 DPI the test had serum and CSF sensitivity of at least 70 and 40%, respectively, for different cutoff values (20, 40, and 80). In trial 1, all control horses maintained serum and CSF titers equal to or below the initial dilutions throughout the study. In contrast, in trial 2, 1 of the 2 control horses had serum (and CSF) titers ranging between 10 and 80 (<5–20), indicating possible natural exposure to S. neurona. Seroconversion tended to occur earlier than CSF conversion. In trial 1, clinical signs among infected horses varied from mild to moderate and appeared between 8 and 29 DPI in 19 infected horses and in 1 of the control horses; histologic CNS lesions were detected in 4 infected horses (Sofaly et al., 2002). In trial 2, clinical signs among infected horses varied from equivocal to mild and were more evident in immunosuppressed horses after 40 DPI; CNS histologic lesions were detected in 7 of 8 infected horses (Cutler et al., 2001). No parasite was observed or isolated from any infected horse in either trial (Cutler et al., 2001; Sofaly et al., 2002). The lack of typical EPM disease and isolation of parasites from the experimentally infected horses, associated with a serologic response of similar magnitude as in the case of the naturally infected horses, indicates the possibility of false-positive results when diagnosing neurological disease in horses exposed to S. neurona but without EPM.

In vaccinated horses the highest serum and CSF titers were obtained at 14 and 28 DPV2. At these time points vaccinated horses had serum and CSF antibody titers that could interfere with the diagnosis of EPM. At 112 DPV2 the titers for all vaccinated horses decreased to values comparable with those of noninfected horses or horses before the experimental challenge.
Therefore, the gain in specificity, and consequently diagnostic certainty, obtained by adding CSF testing to the EPM diagnostic workup might not be as great as expected.

In the present study, titer-specific LRs for the IFAT were estimated for the naturally infected horses. LRs are stable parameters and, like sensitivity and specificity, should be used in conjunction with disease prevalence information (pretest probability) to obtain the probability of disease (post-test probability or titer-specific predictive value) given a specific test result (Dujardin et al., 1994). In addition to eliminating the need for a specific cutoff titer value, titer-specific LRs allow different titer values to have different weights in clinical decisions (Dujardin et al., 1994; Duarte et al., 2003). For instance, for a pretest probability of 25%, a horse with an IFAT serum titer of 20 has approximately a 35% post-test probability of EPM compared with a 92% posttest probability for a horse with a serum titer of 160. Similar calculations are not possible for the WB tests because test results are not quantitative. Hence, titer-specific LRs broaden the possibilities for test interpretation and can potentially improve clinical decision making. In the above example the pretest probability was based on a previously published estimate (Daft et al., 2002), and the post-test probabilities were calculated based on the relationship: post-test odds = titer-specific LR × pretest odds (Dujardin et al., 1994). As a general rule, for a given pretest probability, LRs and post-test probabilities increase as titer values increase (Simel et al., 1993; Dujardin et al., 1994).

Decision analysis based on information on prevalence of the disease, LRs, and correlation between serum and CSF test results should be used to assess the optimal diagnostic protocol for EPM (Smith and Slenning, 2000). However, such analysis is beyond the scope of the present article. Veterinarians, therefore, should consider test correlation when requesting and interpreting multiple diagnostic tests for EPM and take advantage of the benefits of the quantitative nature of the IFAT, where titer values interpreted based on titer-specific LRs might broaden the scope of clinical decisions. For instance, clinicians might decide to treat neurological horses on the basis of high-serum IFAT titers alone, whereas they might decide on a spinal tap in horses with low-serum titers.

In naturally and experimentally infected horses, we found a stronger positive correlation between serum and CSF titers than in vaccinated horses. The ratio of serum to CSF titers for the naturally and experimentally infected horses was consistently smaller than the ratio for the vaccinated horses, which is probably attributable to intrathecal production of antibodies in response to the presence of S. neurona in the CNS. However, no parasite was detected in the CNS of the experimentally infected horses.

The use of C-values and antibody index, 2 forms of weighted serum and CSF antibody ratios, has been suggested to aid in the diagnosis of EPM (Furr, 2002). In contrast to the commonly used immunoglobulin-G index (IgG index), these 2 measures are calculated based on antigen-specific antibody concentration, making them potentially more specific in determining intrathecal production of antibodies. A preliminary evaluation using EPM+ horses and vaccinated horses at 28 DPV2 suggested that a ratio of serum to CSF ≤16 discriminated well between infected and exposed horses (100% sensitivity, 95% specificity). Therefore, there is a potential for the use of different forms of IFAT serum to CSF titer ratios (unadjusted ratio, C-values, an-
In conclusion, the IFAT was reliable and accurate using serum and CSF for the diagnosis of EPM, although the use of S. neurona vaccine might lead to false-positive results in horses vaccinated within the previous 112 days. For clinical diagnosis, the use of titer-specific LRs rather than the traditional measures of sensitivity and specificity has the potential to improve clinical decision making. Furthermore, the correlation between serum and CSF titers can interfere with the joint accuracy of the IFAT. Therefore, the ratio of serum to CSF titers has potential diagnostic value for EPM and should be further investigated.

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