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Comparison of a serum indirect fluorescent antibody test with two Western blot tests for the diagnosis of equine protozoal myeloencephalitis

Paulo C. Duarte, Barbara M. Daft, Patricia A. Conrad, Andrea E. Packham, Ian A. Gardner

Abstract. A serum indirect fluorescent antibody test (IFAT) was compared with a Western blot (WB) and a modified Western blot (mWB) for diagnosis of equine protozoal myeloencephalitis (EPM). Using receiver-operating characteristic (ROC) analysis, the area under the curve of the IFAT was greater than the area under the curves of the WB and the mWB ($P = 0.025$ and $P = 0.044$, respectively). There was no statistically significant difference between the areas under the curves of the WBs ($P > 0.05$). On the basis of an arbitrarily chosen cut-off titer for a positive test result of 1:80 for the IFAT and interpreting weak positive WB results as positive test results, the sensitivities and 95% confidence intervals (CI) of all 3 tests were identical and equal to 88.9% (51.8–99.7%). The specificities and 95% CIs of the IFAT, WB, and mWB test were 100% (91–100%), 87.2% (72.6–95.7%), and 69.2% (52.4–83%), respectively. The overall accuracy of the IFAT was shown to be better than that of the WBs and, therefore, the test has potential for use in the diagnosis of EPM caused by *Sarcocystis neurona*.

Equine protozoal myeloencephalitis (EPM) is a common neurological disease of horses in North America.^{13,19} *Sarcocystis neurona* and *Neospora hughesi* are the protozoal parasites associated with EPM;^{7,17} however, serologic surveys have indicated that exposure to *S. neurona* is more frequent.^{1–3,21,26,27} The seroprevalence of *S. neurona* in US horses has been estimated to be between 34% and 60% in different parts of the country.^{1,2,21,23,26,27}

The Western blot (WB) test has been the standard diagnostic test used for serologic surveys and for the diagnosis of clinically suspected EPM, caused by *S. neurona*. The WB detects specific IgG antibodies in serum and spinal fluid (CSF) of horses infected or exposed to the parasite.¹⁰ The overall sensitivity and specificity of the WB in CSF were estimated to be 87% and 56%, respectively.⁵ In serum, the WB sensitivity and specificity were estimated as 80% and 38%, respectively, for horses with neurologic signs, and 88% and 56%, respectively, for horses without neurologic signs.⁵ The low specificity of the test makes interpretation of a serologic positive test result difficult. To date, a negative WB test result on serum is

the most informative test result for decision-making purposes.⁵

Currently, there is a need for more specific, less labor intensive and less expensive diagnostic tests for EPM. A modified Western blot test (mWB) for detection of equine serum antibodies to *S. neurona* was recently developed.²² The test was reported to have an improved specificity because blots were treated with bovine anti-*Sarcocystis cruzi* antibodies before the addition of equine serum. On the basis of 6 *S. neurona* culture-positive horses (positive gold-standard) and 45 horses living in the eastern hemisphere and assumed never exposed to the parasite (negative gold-standard), the sensitivity and specificity of the mWB were 100% and 98%, respectively.²²

The indirect fluorescent antibody test (IFAT) has been evaluated and used for the diagnosis of protozoal diseases in many species, including horses, where the IFAT has been used to diagnose infections with *Neospora*, *Babesia*, and *Trypanosoma* species.^{3,4,12,15,20,27–29} However, cross-reactivity between *S. neurona* and other species of *Sarcocystis* infecting horses was thought to preclude the use of the IFAT for the diagnosis of EPM.¹⁰

The objective of the present study was to compare the overall accuracy of the IFAT with two different WBs using sera from horses with and without protozoal myeloencephalitis associated with *S. neurona* infection.

Material and methods

Gold standard samples

A total of 109 equine serum samples were available and obtained from horses previously enrolled in a val-

From the Department of Medicine and Epidemiology, School of Veterinary Medicine, One Shields Avenue, University of California, Davis, CA 95616-8737 (Duarte, Gardner), the California Animal Health and Food Safety Laboratory System, University of California, Davis, School of Veterinary Medicine, San Bernardino Branch, San Bernardino, CA 92408 (Daft), and the Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, One Shields Avenue, University of California, Davis, CA 95616-8739 (Conrad, Packham).

validation study of the WB test. Horses were submitted to the California Animal Health and Food Safety Laboratory System for necropsy between 1996 and 1999 as part of the California Horse Racing Board post-mortem program and from the general California horse population. Samples of brain, cardiac and skeletal muscles, and the entire spinal cord were collected, fixed in 10% buffered formalin, and routinely processed for histologic examination of hematoxylin and eosin-stained sections. Histological transverse sections of brain and spinal cord included: thalamus and cerebrum unilaterally; cerebellum; brain stem at rostral colliculus, cerebellar peduncles, mid medulla and obex; cervical segments 1, 3–7; thoracic segments 1, 3, mid and distal; lumbar segments 3–6; and 2 sacral sections. After the initial microscopic screening, fixed brain and spinal cord were transversely sliced at 3–5 mm intervals and reexamined for affected areas. Additional sections were selected if lesions were suspected from gross appearance of sections. Using rabbit anti-*S. neurona* as the primary antibody and the avidin-biotin-immunoperoxidase complex (ABC) system,^a immunostaining for *S. neurona* was performed on central nervous system (CNS) sections having microscopic lesions compatible with EPM. Twelve of these 109 horses were diagnosed as EPM cases and classified as gold-standard positive, whereas the remaining 97 horses were diagnosed as being free of EPM and classified as gold-standard negative. Gold-standard positive horses were horses with histologic lesions compatible with *S. neurona* infection and parasites demonstrated in lesions by immunostaining. Gold-standard negative horses were horses without histologic lesions, insufficient lesions to be considered as EPM suspect, or with non-*Sarcocystis* sp. protozoal parasites identified in the CNS sections.

Of the 109 samples, 48 were selected for inclusion in the present evaluation and were tested by the IFAT, WB, and mWB. The samples were chosen on the basis of availability of serum and targeting a pool of samples from horses with and without neurological disease. Horses with neurological disease were defined as horses with a clinical history of neurologic signs of any form, including performance problems or lameness suspected to be attributable to EPM. Horses without neurological disease included horses that died suddenly, were euthanized for a catastrophic musculoskeletal injury during racing or training, died during surgical repair of acute musculoskeletal injuries, or were euthanized because of irreversible conditions, such as pleuropneumonia, colitis, laminitis, or chronic musculoskeletal disease.

Of the 48 samples, 9 were from gold-standard positive horses (7 neurological, 2 nonneurological) and 39 were from gold-standard negative horses (19 neuro-

logical, 20 nonneurological). Thoroughbreds comprised 52% of the 48 samples tested, whereas Quarter Horses and other breeds represented 25% and 23% of the samples, respectively. Forty-two percent of the horses were females, 45% were geldings and 13% were intact males. The median age of the horses was 4 yr (range, 2–27 yr).

Serum samples were collected by practitioners in the field or by laboratory personnel, upon arrival of the carcass at the laboratory. The maximum time between death and sample collection was 3.5 hr. All serologic tests used in this study were run without the reader's knowledge of the true EPM infection status of the horse tested.

Indirect fluorescent antibody test

Merozoites of the UCD-1 *S. neurona* isolate were used as the test antigen.¹⁶ Parasites were maintained in vitro on a stationary monolayer of MA104 (Rhesus Monkey kidney) cells, harvested by scraping, filtered in a PD-10 Sephadex^b column to minimize cell debris, added to 12-well slides^c in 10 µl aliquots per well, and allowed to air dry. There were approximately 20,000 merozoites per well. Once dry, antigen slides were fixed in 10% formalin for 10 min, washed 2 times in phosphate-buffered saline (PBS), air-dried again, and stored at -70 C until used. Fluorescein-labeled (FITC), affinity-purified antibodies directed against horse-specific IgG^d were diluted 1:1,000 in PBS and added in 10 µl aliquots to each well.

Serum dilutions started at 1:5 and the endpoint titer was the last serum dilution showing distinct, whole parasite fluorescence.⁴ If no fluorescence was evident at a 1:5 dilution, horses were classified as having a titer of <1:5. Each slide was read by one of the authors, experienced with the technique, and by a laboratory assistant in training.

Western blots

The WB and mWB were performed in a private^e and in a state laboratory,^f respectively, as previously described.^{10,22} In brief, blots of the mWB were treated and incubated with fractionated bovine anti-*S. cruzi* IgG antibody diluted at 1:65 in blocking buffer from highly *S. cruzi*-positive cattle (>1:200 in the IFAT) for 1.5 h before the addition of horse serum.²² Merozoites of the SN-3 and MIH1 *S. neurona* isolates were used as antigens by the private and state laboratory, respectively.

Results for the WB test were based on reactivity with the 16 kD molecular weight antigens, and reported as negative, weak positive, positive, or positive with high reactivity. The results for the mWB test were based on reactivity to antigens of 16 and 30 kD and reported as negative, suspect (reaction to either 16 kD

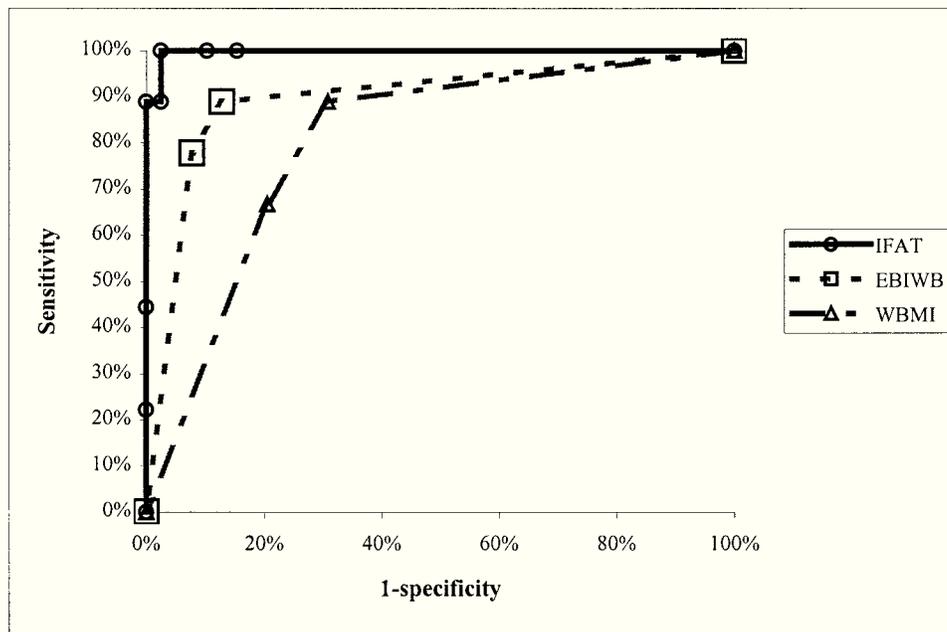


Figure 1. Receiver operating characteristic curves for the serum IFAT, WB, and mWB tests for diagnosis of EPM.

or 30 kD antigens), weak positive, and positive. To allow direct comparison between the WB and mWB data, WB positive and positive with high reactivity results were grouped together, and mWB suspects and negative results were also combined in one group.

Statistical analysis

Receiver-operating characteristic (ROC) analysis was used to compare the overall performance of the IFAT and the two WBs.¹¹ The sensitivity and specificity of the tests were calculated based on an arbitrarily chosen cut-off point for a positive test result. Sensitivity, specificity, and exact binomial 95% confidence intervals (CI) were calculated for each test using standard formulas.²⁵ The agreement between the IFAT results recorded by the two different readers was used as a measure of interobserver test reliability. Based on the arbitrarily chosen cut-off titer for a positive test result, horses in each gold standard group were classified as IFAT positive or negative. The percentage of agreement was calculated for each gold-standard group and an overall kappa statistic was calculated as a measure of agreement beyond chance.¹⁴ Differences in recorded titer values between readers were described. Only the IFAT results recorded by the most experienced reader were used for test comparison purposes.

The software CMDT,^g Epi Info,^h and MedCalcⁱ were used for data analysis.

Results

The median IFAT titer for the gold-standard positive group was 1:80 (range, 1:20 to 1:320). The 2 nonneu-

rological gold-standard positive horses had titers of 1:20 and 1:80. The median IFAT titer for the gold-standard negative group was <1:5 (range, <1:5 to 1:40). The areas under the ROC curves (AUC) and their 95% CIs for the IFAT, WB, and mWB were 0.997 (0.92, 1), 0.896 (0.774, 0.965), and 0.802 (0.662, 0.903), respectively. The AUC of the IFAT was significantly greater than the AUCs of the WB and mWB ($P = 0.025$ and $P = 0.044$, respectively). There was no statistically significant difference between the AUCs of the WBs ($P > 0.05$) (Fig. 1).

On the basis of the ROC analysis, maximal IFAT sensitivity and specificity were obtained at cut-off titers of 1:20 and 1:80, respectively. The highest sensitivity and specificity for both WBs were achieved when weak positive test results were grouped with positive test results. Using a titer of 1:80 as the cut-off for the IFAT and grouping the weak positive with the positive results for the WBs, the sensitivities and 95% CIs of all 3 tests were identical and equal to 88.9% (51.8–99.7%). The specificities and 95% CIs of the IFAT, WB, and mWB test were 100% (91–100%), 87.2% (72.6–95.7%), and 69.2% (52.4–83%), respectively.

Five of the 39 gold-standard negative horses were diagnosed with non-*S. neurona* protozoal encephalitis. Two of these horses were WB positive (1 weak positive) and 3 were mWB positive (1 weak positive). Four of these horses had IFAT titers <1:5 and 1 had a titer of 1:10. None of the 5 horses had neurological disease. Among the gold-standard negative horses there was 1 that had muscle sarcocysts reported. There was no fur-

ther investigation to identify the species of *Sarcocystis*. This horse was negative in both WBs and had an IFAT titer <1:5.

The interobserver agreement for the IFAT results was 78% (7/9) for the gold-standard positive group and 97% (38/39) for the gold-standard negative group. The overall kappa statistic and its 95% CI were 0.763 (0.508, 1). Endpoint titers recorded by the less experienced reader were 1 dilution lower in 4 of 9 horses in the gold-standard positive group and 1 dilution higher in 1 of 39 horses in the gold-standard negative group.

Discussion

Cross-reactivity between *S. neurona* and other species of *Sarcocystis* infecting horses was thought to preclude the use of the IFAT for the diagnosis of EPM.¹⁰ In this study, however, the overall accuracy of the IFAT was shown to be better than that of the WBs and the test discriminated well between horses with and without EPM. The sensitivities and specificities of the tests were estimated using a cut-off titer of 1:80 for the IFAT and grouping the weak positive test results with the positive test results for the WBs. The choice of a specific cut-off point for the IFAT and the criteria for interpretation of weak positive test results for the WBs was arbitrary because it should be based on factors such as the purpose of the testing, the prevalence of the disease, and the costs of misclassification. Therefore, the most appropriate cut-off point (titer) might vary in different circumstances. Higher cut-off titer values for the IFAT maximize specificity, whereas lower cut-off titer values maximize sensitivity. Similarly, interpreting WB weak positive test results as positives increase sensitivity, whereas interpretation of weak positives as negative test results increase specificity. Quantitative tests such as the IFAT have the advantage that choosing a cut-off point for a positive test result is not necessary because titer-specific likelihood ratios can be used for interpretation of test results.⁹ Likelihood ratios express the likelihood that a certain titer value comes from a horse with EPM compared with the likelihood that the same titer value comes from a non-EPM horse. The small sample size precluded the estimation of titer-specific likelihood ratios in the present report.

The experience of the reader is an important factor influencing the results of all 3 tests because there is a certain degree of subjectivity in the reading of test results. There have been no studies assessing the interobserver reliability of the WB for the diagnosis of EPM. We found differences in the IFAT results between readers with different experience. However, the high kappa value between the two readers indicated high interobserver reliability of the IFAT.¹⁴ Only the

IFAT results recorded by the most experienced reader were used for test comparison purposes in the present report.

In this study, comparison of test accuracy was based on a panel of sera from horses of known EPM status. There was careful and extensive examination of the CNS of the gold-standard horses, particularly the spinal cord and brainstem, the most common areas for EPM lesions.⁸ Therefore, it seems unlikely that existing CNS lesions were not detected and misclassification of the gold-standard negative horses occurred. However, it is possible that some of the gold-standard negative horses were sampled early in the course of infection, before any CNS lesions could be detected but after an antibody response to the parasite had occurred. To date, there has been no evidence of *S. neurona* in equine tissues other than the CNS.^{8,24} Experimental infection studies in mice and horses have suggested limited multiplication of the parasite in tissues other than the CNS, fast migration of the parasite to CNS tissues after infection, and evidence that seroconversion and clinical signs in horses, occur as early as 9 days after infection.^{6,24}

The gold-standard negative horses were all from United States, and hence may have been exposed to *S. neurona* and developed serum antibodies against the parasite without CNS infection. Such horses would have been classified as false positives on the basis of our gold-standard definition. However, this classification was deemed appropriate for the purpose of our study. False-positive results might have been attributable to vaccination against EPM. However, horses were enrolled in the study between 1996 and 1999 and the EPM vaccine was first conditionally licensed in the United States in December 2000. Therefore, vaccination was not expected to be a likely source of false-positive results in the present study.

Apparently, cross-reactivity between *S. neurona* and *S. fayeri*, another species of *Sarcocystis* infecting horses, is not a factor in WB false-positive results.¹⁰ However, the extent of WB cross-reactivity between *S. neurona*, other species of *Sarcocystis* infecting horses (*S. equicanis*, *S. bertrani*), and other apicomplexan parasites is not clear. In this study, we found gold-standard negative horses diagnosed with non-*S. neurona* protozoal encephalitis to be positive in the WB and mWB test. This could indicate cross-reactivity between *S. neurona* and other protozoa infecting horses, possibly *N. hughesi*, or simply, exposure to *S. neurona* with antibody production but CNS infection with other protozoa. All 5 horses in this group had low IFAT titers ($\leq 1:10$).

Other factors associated with the parasite itself and the WB technique could affect the accuracy of the WBs. A recent study reported potential antigenic var-

iation among different isolates of *S. neurona*, evaluated by WB analysis.¹⁸ The authors found variation in the intensity of reaction of equine serum to 5 different *S. neurona* isolates (UCD 1–3, SN2, and MU1) but the WB band reaction pattern was essentially the same for all isolates. Differences in intensity of reaction are more likely to affect the sensitivity of the test because weaker WB reactions would be expected when the isolate used in the test is different from the actual field isolate. In the present study, different isolates of *S. neurona* were used in all 3 tests.

Another potential source of variation in accuracy between WBs performed in different settings is variation in the antigen band pattern used to classify a test as positive or negative. The use of reactivity to the 16 and 30 kD antigens as diagnostic criteria for the mWB, in addition to the grouping of suspect results with negative results, increased the specificity estimate for this test.

Using the criteria for a positive and negative test result described above, the sensitivity and specificity estimates for the mWB were lower than the estimates previously reported.²² In the previous study, the gold-standard negative samples were from horses from the Eastern hemisphere, and were assumed to be truly negative because *S. neurona* infections have not been reported in that region of the world.²² The true status of the gold-standard negative horses could not be verified by histological examination and the authors reported that no statistically significant diagnostic criteria for a positive test result could be established, when the blots were not treated with anti-*S. cruzi* antibodies.²²

In conclusion, we demonstrated that the overall accuracy of the IFAT was better than that of the WBs and, therefore, the test has potential for use in the diagnosis of EPM caused by *S. neurona*. The IFAT is faster, less expensive, and less labor intensive than the WB test, and its quantitative nature is an additional advantage because it may be possible to use titer-specific likelihood ratios to aid in the interpretation of test results.

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Sources and manufacturers

- a. ABC-HP Elite, Vector Laboratories, Burlingame, CA.
- b. Pharmacia Biotech, Uppsala, Sweden.
- c. Cell Line Associates, Newfield, NJ.
- d. Jackson ImmunoResearch, Inc., West Grove, PA.
- e. Equine Biodiagnostics Inc., University of Kentucky, KY.

- f. Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, MI.
- g. CMDT, version 1.0 β , Institut für Parasitologie and Tropenveterinärmedizin, Freie Universität Berlin, Germany.
- h. Epi Info, version 6.04, Centers for Disease Control and Prevention, Atlanta, GA.
- i. MedCalc, version 6.11, MedCalc software, Broekstraat, Belgium.

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