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Protozoal coinfection in horses with equine protozoal myeloencephalitis in the eastern United States

Sarah Schale[1] | **Daniel Howe[2]** | **Michelle Yeargan[2]** | **Jennifer K. Morrow[3]** |
Amy Graves[3] | **Amy L. Johnson[4]**

[1]Department of Clinical Studies, College of
Veterinary Medicine, Oregon State
University, Corvallis, Oregon

[2]Maxwell H. Gluck Equine Research Center,
University of Kentucky, Lexington, Kentucky

[3]Equine Diagnostic Solutions, LLC,
Lexington, Kentucky

[4]Department of Clinical Studies, New Bolton
Center, School of Veterinary Medicine,
University of Pennsylvania, Kennett Square,
Pennsylvania

Correspondence
Sarah Schale, College of Veterinary
Medicine, Oregon State University, 158
Magruder Hall, Corvallis, OR 97330.
Email: schales@oregonstate.edu

Background: Infection by 2 or more protozoa is linked with increased severity of disease in marine mammals with protozoan encephalitis.

Hypothesis/Objectives: To assess whether horses with equine protozoal myeloencephalitis (EPM) caused by *Sarcocystis neurona* also have evidence of infection with *Neospora hughesi* or *Toxoplasma gondii*. We hypothesized that horses with EPM would be more likely than horses with cervical vertebral stenotic myelopathy (CVSM) to be positive for antibodies to multiple protozoan parasites.

Animals: One hundred one horses with neurologic disease: 49 with EPM and 52 with CVSM.

Methods: Case review. Archived serum and cerebrospinal fluid (CSF) from 101 horses were examined. Inclusion criteria included neurologic disease, antemortem or postmortem diagnosis of EPM or CVSM, and availability of serological results or archived samples for testing. Additional testing for antibodies was performed on serum for *T. gondii*, as well as serum and CSF for *N. hughesi*.

Results: Horses with EPM were more likely than horses with CVSM to have positive immunologic results for *S. neurona* on serum (95.9% versus 76.9%, $P = .0058$), CSF (98.0% versus 44.2%, $P < .00001$), and serum : CSF titer ratio (91.8% versus 0%, $P < .00001$). Positive results for *Neospora* and *Toxoplasma* were uncommon, with total seroprevalence rates of 12.9% and 14.9%, respectively. The proportions of EPM cases testing positive for *Neospora* and *Toxoplasma* (16% and 12%) were not different from the proportions of CVSM cases testing positive (10% and 17%, $P = .31$ and .47, respectively).

Conclusion: Results do not indicate an important role for protozoal coinfection in EPM in the eastern United States.

KEYWORDS

Neospora hughesi, neurology, polyparasitism, *Sarcocystis neurona*, *Toxoplasma gondii*

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; CVSM, cervical vertebral stenotic myelopathy; EPM, equine protozoal myeloencephalitis; IFAT, indirect fluorescent antibody test; NhSAG1, *N. hughesi* surface antigen 1; PM, postmortem; SnSAG2, 4/3-*S. neurona* surface antigen 2, 4/3.

1 | INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a neurologic disease of horses caused primarily by *Sarcocystis neurona* and occasionally by *Neospora hughesi*. Most horses exposed to these protozoans do not have

clinical disease, but a small percentage develop myeloencephalitis.[1–4] Factors that modulate disease progression after infection with *S. neurona* are poorly understood, and might include coinfection with other microorganisms.

Coinfection of 1 host with multiple parasites influences the immune response of the host as well as the pathogenic severity of the parasites.[5–7] However, studies evaluating parasite coinfections in humans and animal models are inconsistent in directly correlating coinfection with severity of signs.[8,9] A recent study found that polyparasitism, specifically looking at coinfections of *S. neurona* and *Toxoplasma gondii* in marine mammals, was associated with increased severity of signs of neurologic disease and postmortem lesions.[10]

Neospora hughesi and *T. gondii* are potential coinfections of protozoal myeloencephalitis in the horse. In contrast to the relatively widespread seroprevalence of *S. neurona*, seroprevalence of *N. hughesi* is much less common, and was recently found in only 2% of potential EPM cases and 34% of healthy horses tested within the United States.[11,12] Because of the rarity of confirmed *N. hughesi* cases resulting in EPM,[13–15] much of the published literature on EPM is focused on *S. neurona*.[3,4,16,17] Seroprevalence of *T. gondii* has been examined in healthy populations of horses worldwide in conjunction with *S. neurona* and *N. hughesi*, and varies from <1% to 34% depending on geographic location.[18–20] A recent study in horses from California found that horses with signs of neurologic disease compatible with EPM were more likely to be seropositive to *T. gondii* compared with nonneurologic horses.[21] To the author's knowledge, cerebrospinal fluid (CSF) samples have not been concurrently assessed with serum samples from horses with neurologic deficits for the presence of antibodies against these protozoa, nor have horses from the eastern United States been assessed.

The aim of our study was to assess whether horses previously diagnosed with EPM caused by *S. neurona* also had evidence of infection with *N. hughesi* or *T. gondii*. We hypothesized that horses with EPM would be more likely than horses with cervical vertebral stenotic myelopathy (CVSM) to be positive for antibodies to multiple protozoal parasites.

2 | MATERIALS AND METHODS

2.1 | Case selection

Paired serum and CSF samples from 101 horses were used for our study. These horses were presented to the George D. Widener Hospital for Large Animals at New Bolton Center for neurologic evaluation over a 6-year period (March 2010 to November 2016). Serum and CSF samples were initially collected for diagnostic purposes, including *S. neurona* and occasionally *N. hughesi* immunologic analysis. After sample collection and initial testing the remaining serum and CSF samples were stored at −80°C until analysis for our study.

Cases were categorized as EPM or CVSM. Each category was subdivided into confirmed cases and presumptive cases depending on whether postmortem confirmation of diagnosis was available. Confirmed EPM cases had clinical history, neurologic deficits, and postmortem lesions consistent with EPM. The pathologic criteria included

multifocal or focally extensive lymphocytic, lymphohistiocytic, or lymphoplasmacytic myelitis, encephalitis, or both. Occasionally, additional confirmatory tests such as immunohistochemistry or PCR tests for *S. neurona* were used at the discretion of the university pathologists. Presumptive EPM cases had clinical history and neurologic deficits consistent with EPM, exclusion of other likely diseases by appropriate diagnostic testing, and SnSAG2, 4/3 ELISA serum : CSF titer ratios ≤50. Confirmed CVSM cases had clinical history, neurologic deficits, and postmortem lesions consistent with CVSM. The pathologic criteria included axonal degeneration and demyelination consistent with spinal cord compression. Nineteen out of 23 (83%) of these cases also had myelographic studies consistent with spinal cord compression, and all had SnSAG2, 4/3 ELISA serum : CSF titer ratios ≥100 with a normal specific index. Presumptive CVSM cases had histories and neurologic deficits consistent with CVSM, myelographic studies indicative of spinal cord compression at 1 or more sites, and SnSAG2, 4/3 ELISA serum : CSF titer ratios ≥100 with a normal specific index.

Although necropsies were not performed in all cases, horses were excluded from the study if the necropsy findings did not support the antemortem diagnosis. Horses were also excluded if inadequate sample volumes were available to perform all immunologic tests.

2.2 | Antibody testing

Testing for antibodies against *S. neurona* (SnSAG 2, 4/3 ELISA; SnSAG 2, 4/3, and NhSAG1 ELISA were performed at Equine Diagnostic Solutions, Lexington, Kentucky) was performed at the time of initial collection for obtainment of a clinical diagnosis, and results were collected from medical records. Nineteen cases were also tested for antibodies against *N. hughesi* (NhSAG1 ELISA) as part of the initial neurologic evaluation; this additional testing was performed at the attending clinician's discretion.

All samples were submitted for testing for antibodies against *N. hughesi* (NhSAG1 ELISA; SnSAG 2, 4/3, and NhSAG1 ELISA were performed at Equine Diagnostic Solutions) if not previously performed.[14] All samples were submitted for detection of antibodies against *T. gondii* via western blot (Western blot analysis was performed at M.H. Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky).[22–26] Samples were considered positive for *T. gondii* if there was evidence of antibody reactivity to the immunodominant major tachyzoite surface antigen SAG1.

2.3 | Statistical analysis

Immunologic results were dichotomized as positive or negative for antibodies against each protozoan. The proportions of positive horses in each group were compared using the "N-1" Chi-squared test.[27,28] A *P* value of < .05 was used to determine statistical significance.

3 | RESULTS

A total of 101 horses were included in the study. Two cases of the original 103 were excluded from analysis because 1 case did not have

TABLE 1 Antibody test results

	All EPM[a] (n = 49) n (%)	All CVSM[a] (n = 52) n (%)	P value[a]	PM EPM[b] (n = 20) n (%)	PM CVSM[b] (n = 23) n (%)	P value[b]
S. neurona						
+Serum	47 (96%)	40 (77%)	.0058	18 (90%)	16 (70%)	.10
+CSF	48 (98%)	23 (44%)	<.00001	19 (95%)	10 (44%)	.00030
+Ratio	45 (92%)	0 (0%)	<.00001	16 (80%)	0 (0%)	<.00001
N. hughesi						
+Serum	8 (16%)	5 (10%)	.31	5 (25%)	3 (13%)	.31
+CSF	2 (4%)	0 (0%)	.14	2 (10%)	0 (0%)	.12
+Ratio	1 (2%)	0 (0%)	.30	1 (5%)	0 (0%)	.28
T. gondii						
+Serum[c]	6 (12%)	9 (17%)	.47	3 (15%)	7 (30%)	.23

[a]Columns display data for all EPM and CVSM cases (confirmed + presumptive) and relevant statistical analyses.
[b]Columns display data only for EPM and CVSM cases that were confirmed on postmortem evaluation, with relevant statistical analyses.
[c]Positive serum results for *T. gondii* were only weak or very weak positives; no strong positive results were obtained.
Abbreviations: EPM, equine protozoal myeloencephalitis; PM, postmortem; CVSM, cervical vertebral stenotic myelopathy; +, positive test result; CSF, cerebrospinal fluid; ratio, serum : CSF titer ratio.

T. gondii results recorded, and the other case was positive for both EPM and CVSM.

Forty-nine cases were categorized as EPM, with 29 considered presumptive and 20 confirmed on postmortem examination. One of the EPM cases in this group was diagnosed with *N. hughesi* infection on the basis of immunologic testing and postmortem examination, and the remaining 48 cases were diagnosed with *S. neurona* infection. Fifty-two cases were categorized as CVSM, with 29 considered presumptive and 23 confirmed on post-mortem examination.

As anticipated, the vast majority (>90%) of EPM cases were positive for *S. neurona* on serum, CSF, and serum : CSF titer ratio (Table 1). Serum titers were variable, with a range from negative (<1 : 250) to 1 : 4000 (median titer 1 : 500). The majority of CVSM cases (76.9%) were also positive for *S. neurona* on serum, though less than half were positive on CSF and none on serum : CSF titer ratio. Serum titers for the CVSM cases were also variable, with a range from negative (<1 : 250) to 1 : 4000 (median titer 1 : 500). Equine protozoal myeloencephalitis cases were significantly more likely than CVSM cases to be positive for *S. neurona* on serum (P = .0058), CSF (P < .00001), and serum : CSF titer ratio (P < .00001).

Overall, 12.9% of all cases were positive on serum for *N. hughesi*. The proportion of EPM cases that tested positive did not differ from the proportion of CVSM cases (P = .31). Only 1 EPM case showed evidence of intrathecal antibody production with a positive serum : CSF titer ratio, and this case was confirmed to have *N. hughesi* rather than *S. neurona* on necropsy and did not have a positive titer ratio for *S. neurona*. None of the CVSM cases had positive CSF or titer ratio results for *N. hughesi*.

Overall, 14.9% of all cases were weakly or very weakly positive on serum for *T. gondii*. No horse in either group had a serum result considered positive or strongly positive. The proportion of EPM cases that tested weakly positive for *T. gondii* did not differ from the proportion of CVSM cases (P = .47). *Toxoplasma gondii* testing was not performed on CSF samples because of the lack of positive serum results.

4 | DISCUSSION

Our study investigated the prevalence of *T. gondii* and *N. hughesi* infection in conjunction with either EPM or CVSM in horses from the eastern United States. Because antigen detection techniques for these protozoans are generally unavailable or unreliable in clinical cases, antibody tests were used to detect infection. Subclinical infection (exposure) was inferred for horses with positive serum but negative titer ratio results. Clinical infection was assumed for horses that had positive serum : CSF titer ratios and also met the other criteria for EPM diagnosis. Over the 6-year interval studied, no horse was diagnosed with clinical coinfection (ie, positive titer ratio to more than 1 protozoa). Similarly, occurrence of subclinical coinfection with these protozoa was found to be negligible in both groups of horses. Therefore, our data do not support protozoal coinfection as a common finding in horses with neurologic disease from the eastern United States, suggesting that coinfection with protozoan species is unlikely to play an important role in development of clinical disease caused by *S. neurona* infection.

Studies assessing seroprevalence of *S. neurona* and *N. hughesi* in horses in the United States have found variable results in different populations. Seroprevalence of antibodies against *S. neurona* and *N. hughesi* in 5250 healthy horses across the United States was 78% and 34%, respectively.[11] Seroprevalence of *S. neurona* and *N. hughesi* in 3123 horses across the United States with clinical signs compatible with EPM was 27.8% and 2.0%, respectively.[12] Our study population came from a region with high exposure to *S. neurona*, as shown by the 77% seroprevalence in the CVSM group. However, exposure to *N. hughesi* was much less common, with 12.9% of all cases having detectable antibody concentrations.

Toxoplasma gondii is rarely linked to clinical disease in the horse, although it was originally implicated as the causative agent of "segmental myelitis" (now EPM) before *S. neurona* was identified as a distinct protozoan species. Cases of ophthalmic and transplacental infections caused by *T. gondii* occur in horses, but horses appear to be relatively

resistant to clinical disease from *T. gondii* compared with most warm-blooded vertebrates.[29,30] Recently, horses with neurologic disease in California had >6 times odds of being seropositive to *T. gondii* compared with controls (using a titer cutoff of 1 : 320).[21] However, that study did not assess CSF antibody concentrations, serum : CSF titer ratios, or have necropsy confirmation of diagnosis. Therefore, the authors could not assess whether coinfections contributed to development or severity of EPM. Protozoal coinfections, specifically concurrent infections with *S. neurona* and *T. gondii*, are an important cause of encephalitis in marine life.[10] Marine species could be particularly sensitive to protozoal infections, or have increased exposure based on geographic location and contaminated waterways.

Not all cases used for our study had postmortem confirmation of diagnosis. The EPM group was diagnosed using serum : CSF titer ratios or necropsy findings, and the CVSM group diagnosed with myelography or necropsy in addition to negative serum : CSF titer ratios for EPM. The CVSM group established a convenient negative control group from a CSF sampling perspective, because horses undergoing myelography at the George D. Widener Hospital for Large Animals at New Bolton Center routinely have CSF and serum samples collected and stored. As this was a retrospective study from banked samples on client-owned horses, results of postmortem evaluation were not always available. While postmortem examination is the gold standard for diagnosis of protozoal infections and CVSM, cases without this diagnostic were included in our study to incorporate a larger sample size. Because stringent criteria were used for inclusion in the study, we have high confidence in the validity of the cases.

When postmortem examination was performed, organism, or antigen detection techniques such as immunohistochemistry and PCR were not always used. Protozoal organisms can be difficult to identify in clinical cases, which frequently have been treated extensively with antiprotozoal agents before death, thus substantially decreasing the protozoal load. Therefore, the possibility exists that horses with coinfections were missed, because we relied heavily on immunological test results (specifically the serum : CSF titer ratio) to determine cause of infection. However, because of high sensitivity and specificity of the SnSAG2, 4/3 ELISA,[3] as well as the absence of immunological evidence for *N. hughesi* or *T. gondii* infection, we believe that clinical coinfections were not present in this population. Future work should include a larger population of horses from additional geographical regions.

Diagnostic tests for *T. gondii* have not been validated for use with horse samples.[31] Therefore, Western blot was used to detect antibodies against *T. gondii*, which is consistent with prior studies investigating *T. gondii* infection in several other species, including humans, ruminants, and mice.[22–26] Western blot is not a quantitative test, which prevents its use for establishing an end-point titer. However, it is an excellent qualitative assay that provides a visual confirmation of the expected antibody reactivity with known immunodominant antigens, thereby giving high confidence in a positive versus negative result. In our study, cases tested "weakly positive," "very weakly positive," or "negative" on serum, which was deemed clinically insignificant; for this reason, additional testing of CSF was not pursued.

In conclusion, coinfections with either *T. gondii* or *N. hughesi* did not appear to contribute to development of clinical EPM caused by *S. neurona* infections in horses in the eastern United States. The absence of clinically relevant coinfections might be region-specific and related to low equine exposure to protozoa other than *S. neurona*. Other infectious agents, including viruses and bacteria, could also play a role in the development of clinical EPM through either direct effects or immune modulation. Regardless, further work is needed to elucidate the complexities of clinical EPM for the development of additional preventative and treatment strategies.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest with the contents of this article.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

ORCID

Sarah Schale http://orcid.org/0000-0002-8703-6692
Amy L. Johnson http://orcid.org/0000-0003-2507-0040

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