Cerebrospinal fluid Lyme multiplex assay results are not diagnostic in horses with neuroborreliosis

Amy L. Johnson1 | Laura K. Johnstone2 | Darko Stefanovski1

1Department of Clinical Studies, New Bolton Center, University of Pennsylvania School of Veterinary Medicine, Kennett Square, Pennsylvania
2Massey University, Palmerston North, New Zealand

Correspondence
Amy L. Johnson, New Bolton Center, 382 W. Street Rd., Kennett Square, PA 19348. Email: amyjohn@vet.upenn.edu

Background: The accuracy of the Lyme multiplex assay for the diagnosis of neuroborreliosis in horses is unknown.

Hypothesis/Objectives: To describe Lyme multiplex results in horses with a postmortem diagnosis of neuroborreliosis. The hypothesis was that paired serum and cerebrospinal fluid (CSF) results and a CSF : serum ratio would allow differentiation of horses with neuroborreliosis from those with other neurologic diseases.

Animals: Ninety horses that had neurologic examinations, serum and CSF Lyme multiplex analyses, and postmortem examination of the nervous system performed.

Methods: Retrospective study. Data collected included signalment, ante- and postmortem diagnoses, and serum and CSF Lyme multiplex results. The CSF : serum ratio was calculated by dividing CSF median fluorescent intensity (MFI) by serum MFI for each result.

Results: Ten horses had a final diagnosis of neuroborreliosis, 70 were diagnosed with other neurologic diseases, and 10 had no neurologic disease. Not all horses with neuroborreliosis had positive results: 4/10 had at least 1 positive serum result, 5/10 had at least 1 positive CSF result, and 3/10 had at least 1 CSF result 4-fold higher than the corresponding serum result. Results were similar for the 70 horses with other neurologic diseases: 53% had at least 1 positive serum result, 50% had at least 1 positive CSF result, and 16% had at least 1 CSF result 4-fold higher than the corresponding serum result.

Conclusions and Clinical Importance: Positive Lyme multiplex results were common in horses with neurologic diseases and did not adequately differentiate horses with neuroborreliosis from horses with other disorders.

KEYWORDS
Borrelia burgdorferi, borreliosis, immunology, serology

1 INTRODUCTION

Infection with Borrelia burgdorferi, the causative agent of Lyme disease, is common in horses. One study in the northeast United States documented a 45% seroprevalence,1 and a more recent study demonstrated a 33% seroprevalence in southwest Virginia.2 Seropositive horses usually are clinically normal, showing that infection with Borrelia usually causes subclinical or no disease.
Nevertheless, some infected horses go on to develop severe manifestations of disease such as nervous system infection with *B. burgdorferi*, known as neuroborreliosis, an important but rare cause of neurologic disease in horses. Neuroborreliosis is difficult to diagnose, and postmortem histology is considered the most accurate diagnostic technique. Challenges to diagnosis in the living horse are because of the discrepancy between seroprevalence and clinical disease, as well as the variability in clinical signs and laboratory results among affected horses. Reported clinical signs include muscle atrophy or weight loss, cranial nerve deficits, ataxia, behavior changes, dysphagia, muscle fasciculations, neck stiffness, and episodic respiratory distress. These signs are similar to those observed with other, more common, neurologic diseases in horses, and laboratory testing is necessary for accurate diagnosis. However, documented cases have had inconsistent laboratory results regardless of whether culture, polymerase chain reaction (PCR), or immunologic tests were utilized.

Similar difficulties exist in human medicine. The American Academy of Neurology utilizes 3 criteria for the diagnosis of neuroborreliosis, including possible tick exposure in an endemic area, compatible neurologic disease for which other potential etiologies have been excluded, and the presence of ≥1 of the following: skin reaction such as erythema migrans; immunologic evidence of infection, preferentially with documentation of intrathecal antibody production; or, *Borellia* identification by culture, histology, or PCR. Likewise, the European Federation of Neurological Societies uses 3 criteria, including compatible neurologic disease for which other potential etiologies have been excluded, cerebrospinal fluid (CSF) pleocytosis, and intrathecal antibody production. It would be ideal to validate such diagnostic criteria for horses. Because there are many areas where horses could be exposed to *B. burgdorferi* and clinical signs of neuroborreliosis are so variable, documentation of intrathecal antibody production seems an ideal adjunct to diagnosis in the living horse. This concept is already widely utilized in horses for the diagnosis of equine protozoal myeloencephalitis (EPM), and the availability of validated tests that can detect the presence of intrathecal antibody production has improved EPM diagnosis substantially in the last 5 years.

The Lyme multiplex assay is a bead-based multiple antigen immunofluorescent assay that detects antibodies against 3 *B. burgdorferi* outer surface proteins (OspS) that have variable expression. The OspA is primarily expressed within the tick, whereas OspC is expressed during early infection of the mammalian host and OspF during more chronic infection. This assay can be performed on serum and CSF, but its accuracy in the diagnosis of neuroborreliosis has never been assessed. The purpose of our study was to compare Lyme multiplex results on serum and CSF from horses with neuroborreliosis to those from horses with other neurologic diseases, and to assess whether a CSF : serum ratio would allow accurate diagnosis of neuroborreliosis.

## 2 | MATERIALS AND METHODS

Retrospective analysis of medical records was conducted to identify equine patients at a referral hospital (New Bolton Center, University of Pennsylvania School of Veterinary Medicine, Kennett Square, PA) that had a neurologic examination, Lyme multiplex analysis of serum and CSF, and postmortem examination of the nervous system. Signalment, antemortem diagnosis, final postmortem diagnosis, and Lyme multiplex assay results were recorded for each horse. Results for the Lyme multiplex assay were recorded as median fluorescent intensity (MFI) and categorized as negative, equivocal, or positive by the diagnostic laboratory. Cut-offs provided by the laboratory were used for this study (positive cut-offs >2000 MFI for OspA, >1000 MFI for OspC, and >1250 for OspF). According to the diagnostic laboratory, serum samples are diluted 1 : 400 before analysis whereas CSF samples are undiluted.

Horses initially were categorized into 3 groups based on final postmortem diagnosis: neuroborreliosis, other neurologic disease, or no neurologic disease. Postmortem diagnosis of neuroborreliosis was achieved by documenting characteristic histologic lesions as described previously. Each horse had 6 MFI results: serum OspA, serum OspC, serum OspF, CSF OspA, CSF OspC, and CSF OspF. For each pair of Osp results, a CSF : serum ratio was calculated by dividing the CSF MFI result by the corresponding serum MFI result. Ratios >4 were considered positive. This cut-off was chosen based on our experience, discussion with personnel at the testing laboratory, and potential similarity to validated cut-offs used in EPM diagnostic testing. Overall seroprevalence, CSF prevalence, and percentage of horses with positive ratios were calculated for each group and each type of antibody.

Analyses were performed using a commercially available statistical software package (STATA 14 MP, StataCorp, College Station, Texas).

### 3 | RESULTS

Ninety horses met the inclusion criteria. Ten of these horses had a final diagnosis of neuroborreliosis, 70 were diagnosed with other neurologic diseases, and 10 had no neurologic disease identified. Supporting Information Tables 1, 2, and 3 show the demographic information, diagnosis, and Lyme multiplex assay results for horses with neuroborreliosis, other neurologic disease, and no neurologic disease, respectively. Results from the 10 horses with neuroborreliosis have been previously described in the literature. Of the 70 horses with other neurologic diseases, necropsy diagnoses included cervical vertebral stenotic myelopathy (n = 22), EPM (n = 12), degenerative myelopathy (n = 12; includes those categorized as equine degenerative myeloencephalopathy, neuroaxonal dystrophy, or degenerative myelopathy), traumatic injuries (n = 5), and various other neurologic conditions (n = 19). None of the horses had a known history of receiving a Lyme vaccination of any type.

Table 1 summarizes the Lyme multiplex results obtained for horses in this study. Overall seroprevalence was 51% and horses with
neuroborreliosis had similar serologic results to other horses, with positive results in 4/10 with neuroborreliosis, 37/70 (53%) with other neurologic disease, and 5/10 with no neurologic disease. Similarly, the overall CSF prevalence was 47%, with positive results in 5/10 with neuroborreliosis, 35/70 (50%) with other neurologic disease, and 2/10 with no neurologic disease. Using a CSF : serum ratio cut-off of >4, only 18% of all horses were positive, including 3/10 with neuroborreliosis, 11/70 (16%) with other neurologic disease, and 2/10 with no neurologic disease. Across all groups of horses (neuroborreliosis, other neurologic disease, and no neurologic disease) and all sample types (serum, CSF, and CSF : serum ratio), horses were least likely to have positive results for OspA and most likely to have positive results for OspF.

Table 2 shows Spearman correlation results for Lyme multiplex assay variables. The OspC and OspF CSF : serum ratios were most strongly correlated with a neuroborreliosis diagnosis (P = .0854 and .0605, respectively). None of the other Lyme multiplex assay variables were associated with neuroborreliosis. Because CSF : serum ratios appeared correlated, ROC curves were created to assess how well these ratios might perform in a clinical setting. Table 3 and Figures 1 and 2 describe ratio performance. The OspC CSF : serum ratio had an area under the ROC curve (AUC) of 0.333 and therefore showed no discriminatory ability for horses with neuroborreliosis from horses with other disorders. The OspF CSF : serum ratio showed relatively poor discriminatory ability, with AUC of 0.683. Using a probability cut-off of 0.1, the OspF CSF : serum ratio had a sensitivity of 40%, specificity of 79%, positive predictive value of 19%, negative predictive value of 91%, and overall accuracy of 74%.

4 | DISCUSSION
Our results highlight the difficulty of diagnosing neuroborreliosis in the living horse. As mentioned earlier, several challenges exist. The first is that documented clinical disease is rare, whereas subclinical infection

### Table 1: Lyme results using multiplex assay, by postmortem diagnosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Neuroborreliosis (n = 10)</th>
<th>Other neurologic disease (n = 70)</th>
<th>No neurologic disease (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum positive OspA* (%)</td>
<td>1 (10%)</td>
<td>6 (9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Serum positive OspC* (%)</td>
<td>0 (0%)</td>
<td>8 (11%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Serum positive OspF* (%)</td>
<td>4 (40%)</td>
<td>30 (43%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>Serum positive any (%)</td>
<td>4 (40%)</td>
<td>37 (53%)</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>CSF positive OspA* (%)</td>
<td>2 (20%)</td>
<td>3 (4%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>CSF positive OspC* (%)</td>
<td>2 (20%)</td>
<td>17 (24%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>CSF positive OspF* (%)</td>
<td>5 (50%)</td>
<td>32 (46%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>CSF positive any (%)</td>
<td>5 (50%)</td>
<td>35 (50%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Ratio positive OspA* (%)</td>
<td>1 (10%)</td>
<td>3 (4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Ratio positive OspC* (%)</td>
<td>2 (20%)</td>
<td>8 (11%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Ratio positive OspF* (%)</td>
<td>3 (30%)</td>
<td>8 (11%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Ratio positive any (%)</td>
<td>3 (30%)</td>
<td>11 (16%)</td>
<td>2 (20%)</td>
</tr>
</tbody>
</table>

* MFI values > 2000 for OspA considered positive.
* MFI values > 1000 for OspC considered positive.
* MFI values > 1250 for OspF considered positive.
* CSF MFI value : serum MFI value ratio >4 considered positive.

### Table 2: Spearman correlation results for Lyme multiplex assay variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>rho</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OspA serum</td>
<td>-0.0898</td>
<td>.3998</td>
</tr>
<tr>
<td>OspC serum</td>
<td>-0.0225</td>
<td>.8336</td>
</tr>
<tr>
<td>OspF serum</td>
<td>-0.0885</td>
<td>.4070</td>
</tr>
<tr>
<td>OspA CSF</td>
<td>-0.0993</td>
<td>.3515</td>
</tr>
<tr>
<td>OspC CSF</td>
<td>0.1259</td>
<td>.2371</td>
</tr>
<tr>
<td>OspF CSF</td>
<td>0.0973</td>
<td>.3616</td>
</tr>
<tr>
<td>OspA CSF : serum ratio</td>
<td>-0.0014</td>
<td>.9898</td>
</tr>
<tr>
<td>OspC CSF : serum ratio</td>
<td>0.1824</td>
<td>.0854</td>
</tr>
<tr>
<td>OspF CSF : serum ratio</td>
<td>0.1987</td>
<td>.0605</td>
</tr>
</tbody>
</table>

### Table 3: Performance of CSF : serum ratios in diagnosis of neuroborreliosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>ROC AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OspC CSF : serum ratio</td>
<td>0.997</td>
<td>0.850-1.169</td>
<td>0.333</td>
</tr>
<tr>
<td>OspF CSF : serum ratio</td>
<td>1.140</td>
<td>1.003-1.295</td>
<td>0.683</td>
</tr>
</tbody>
</table>

ROC, receiver operating characteristic; AUC, area under the ROC curve; CI, confidence interval. Results from 2 independent univariate logistic regressions where the independent variables are either OspC CSF : serum ratio or OspF CSF : serum ratio.
with the causative organism appears to be widespread in horses living in endemic areas. Seroprevalence studies in normal, healthy horses indicate infection rates of up to 45% in endemic areas, whereas only 23 confirmed cases have been reported in the literature. Some cases may be missed, but even at our hospital, in an endemic area where clinical suspicion of the disease is high, usually only 1–2 new cases are diagnosed annually with postmortem confirmation. The neuroborreliosis cases reported here and previously might represent only the extreme end of the disease spectrum, with clinical signs so severe and unresponsive to treatment that euthanasia was required, permitting definitive diagnosis. Less severe cases might be under-reported because of the tremendous difficulty in confirming that clinical signs in the living horse are caused by Borrelia infection and not another cause. Lack of a reliable antemortem diagnostic test is a major obstacle. Only 2 of the previously reported cases were definitively diagnosed before death, both with positive CSF PCR results for Borrelia. Serologic results for reported cases have been variable and often negative or equivocal, regardless of the assay utilized. Likewise, organism or antigen detection tests such as culture, PCR, and histopathology have yielded inconsistent results.

Similar challenges exist in human medicine, although antemortem diagnosis is more standardized. Cerebrospinal fluid culture for Borrelia is considered a low-yield diagnostic test and generally is restricted to research studies because of the need for special expertise and

FIGURE 1 ROC curve for the performance of an OspC CSF : serum ratio in the diagnosis of neuroborreliosis

FIGURE 2 ROC curve for the performance of an OspF CSF : serum ratio in the diagnosis of neuroborreliosis
equipment.\textsuperscript{14} Cerebrospinal fluid PCR for *Borrelia* is only positive in a small number of patients with late Lyme neuroborreliosis, and does not confirm active infection.\textsuperscript{14} The most accurate laboratory test is identification of *Borrelia*-specific intrathecal antibody production, but this test has low sensitivity in the early phase of disease and positive results may persist for years after eradication of infection; results cannot be used to confirm active infection or assess adequacy of antibiotic treatment.\textsuperscript{14} Therefore, the American Academy of Neurology and the European Federation of Neurological Societies have established the previously described guidelines for definitive antemortem diagnosis of neuroborreliosis.\textsuperscript{4,5} Both sets of guidelines recommend documentation of *Borrelia*-specific intrathecal antibody production as part of the criteria.

Because documentation of intrathecal antibody production is standard practice in the diagnosis of neuroborreliosis in humans and for other infectious nervous system diseases, we attempted to apply similar criteria to horses. This methodology already has been applied to EPM, another infectious nervous system disease, with substantial improvement in diagnostic accuracy.\textsuperscript{6} Previous research in horses indicates that normal horses have passive diffusion of antibody from blood to CSF at a serum : CSF titer ratio > 130 : 1.\textsuperscript{15,16} Based on this information, a method using the Sarcocystis neurona surface antigen ELISAs (SnSAG2 and SnSAG4/3 ELISAs) to determine serum : CSF titer ratios was validated for detection of intrathecal antibody production against *S. neurona* and accurate diagnosis of EPM in horses.\textsuperscript{17} Using this method and a titer ratio cut-off of 100, horses with a titer ratio <100 were 24 times more likely to have EPM; test sensitivity was estimated at 86% and specificity at 96%. For the current study, a similar cut-off was arbitrarily chosen to evaluate the Lyme multiplex assay. According to the testing laboratory, serum samples are tested at a 1 : 400 dilution and CSF samples are tested undiluted. No correction is made for the dilution on the reported MFI results. Therefore, to account for sample dilution, the serum MFI result was multiplied by 400. If a serum : CSF titer ratio cut-off of 100 is used with correction for the dilution factor [(serum MFI result \times 400) : CSF MFI result < 100], the equation can be simplified [serum MFI result \times 4 < CSF MFI result] to state that any CSF MFI result > 4 times the corresponding serum MFI result equals a “positive” result, indicative of intrathecal antibody production. This ratio cut-off was further supported by our experience, where we have noted that horses with disorders other than neuroborreliosis often have CSF MFI results 2–3 times those of their serum MFI results, and discussion with the test developer, who indicated that CSF : serum ratios > 4 are supportive of neuroborreliosis (Dr Bettina Wagner, personal communication 5/14/15). Unfortunately, results of this study do not validate the use of the Lyme multiplex assay in this way for the diagnosis of neuroborreliosis.

Simple calculation of a CSF : serum ratio technique has been successfully implemented for EPM diagnosis, but meningitis is not a common feature of this disease and CSF cytologic results generally are normal. Conversely, most horses with neuroborreliosis have abnormal blood–brain barrier permeability as evidenced by abnormal CSF cytology and marked histologic changes including leptomenigitis and vasculitis. Unfortunately, Lyme multiplex assay results as reported cannot be used to generate a C-value or antibody index. The results are reported as MFI and are not actual titers, and the linear range of the assay has never been established. Relative MFI is calculated using 5-variable logistic regression, which means that the assay is not linear at very low or high antibody concentrations. Serum is diluted at 1 : 400 before analysis, but CSF is not diluted. Because of the methodology of the assay, a simple mathematical correction (ie, multiplying serum MFI by 400) does not appropriately correct for the dilution factor and thus does not allow for accurate comparison to CSF results. Other commercial immunologic tests are available, but they are unlikely to perform more effectively. Both the enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) yield quantitative results, but there are known issues with sensitivity and specificity compared to the multiplex assay.\textsuperscript{7} Western blot does not provide quantitative results and is unsuitable for assessing intrathecal antibody production. Additional research is warranted to investigate more sophisticated methods of assessing intrathecal *Borrelia*-specific antibody production in horses. These might involve using a similar testing platform (multiplex assay) to quantify total IgM/IgG or IgG isotypes in serum and CSF in addition to *Borrelia*-specific antibody and albumin concentrations to allow calculation of a C-value (Goldmann–Witmer coefficient) and antibody index. Alternatively, pre-analytical manipulation of the samples could be used to equalize the total amount of immunoglobulin in serum and CSF aliquots, which then would allow identification of *Borrelia*-specific intrathecal antibody production.

One surprising finding, which has been noted previously in the literature, is the number of horses with neuroborreliosis that have negative immunologic test results. Recent infection with testing performed before seroconversion is a possible explanation; acute Lyme neuroborreliosis occurs in people days to weeks after infection.\textsuperscript{14} However, we have repeated serologic testing on seronegative neuroborreliosis horses and documented their failure to seroconvert months after appearance of clinical signs. Of the 6 seronegative neuroborreliosis horses in this study, 4 had shown neurologic signs for >1 month before testing. Negative serum results could be explained by sequestration of the organism in the central nervous system, with decreased antigen in the rest of the body to a level that failed to stimulate a continued immune response. However, 5/10 horses with neuroborreliosis did not have detectable CSF antibody concentrations, despite evidence of continued CNS infection on postmortem examination. Variation in the spirochete itself could cause these negative results, with other *Borrelia* species or strains or variable expression of Osps failing to stimulate host production of anti-OspA, OspC, or OspF antibodies. *Borrelia burgdorferi* sensu lato comprises 20 different genospecies; 3 (*B. burgdorferi*, *B. afzelii*, and *B. garinii*) are primarily responsible for Lyme borreliosis in humans, with *B. burgdorferi* sensu stricto being the primary cause in the
United States.\textsuperscript{14} All genospecies have a highly conserved linear chromosome but multiple linear and circular plasmids with a high degree of variation. Intraspecies diversity is common; \textit{B. burgdorferi} strains in the United States have been divided into several subtypes based on genetic variation, including genes that encode OspC.\textsuperscript{14} Heterogeneity among strains is assumed to be the main factor causing regional differences in the clinical expression of Lyme borreliosis in humans,\textsuperscript{18} and might be responsible for some of the difficulties in diagnosing neuroborreliosis in horses. Despite using all currently available techniques in the postmortem diagnosis of neuroborreliosis, it is also possible that some cases were misdiagnosed, which could explain failure of antibody detection. Alternatively, perhaps the variation lies not within the organism, but within the equine host. The kinetics of OspA, OspC, and OspF antibody production in the horse have been largely extrapolated from other species and not specifically evaluated after experimental infection. Possibly, the Osps are less immunodominant in horses than in other species, or horses that acquire neuroborreliosis fail to recognize Osps because of variation in immune function. B-cells are required for antibody production, and a previously reported equine case showed evidence of common variable immunodeficiency (CVID), a disease of B-cell maturation that leads to B-cell lymphopenia or depletion and low serum antibody concentrations.\textsuperscript{11} Common variable immunodeficiency or other less well-characterized immunodeficiencies in affected horses could explain both the rarity of neuroborreliosis in horses and the inconsistent immunologic test results. One of the neuroborreliosis cases in our study had a concurrent diagnosis of CVID; this horse was negative for all Lyme multiplex results in both serum and CSF.

False positive results in horses without neuroborreliosis also were common, which was less surprising because of widespread exposure to and infection with the causative organism, anticipated passive diffusion of antibodies into CSF, and the difficulties in establishing whether intrathecal antibody production was present. However, when we reviewed individual cases, some results have been surprising. For example, a horse diagnosed on postmortem examination with \textit{Klebsiella} meningitis, with no histologic evidence of \textit{Borrelia} infection, had negative or low positive serum Lyme multiplex results, with strongly positive CSF Lyme multiplex results that were 14–25 times higher than serum results. Certainly, a potential explanation for false positive results is that the arbitrarily chosen cut-off value was inappropriate. However, we also are suspicious that some horses without neuroborreliosis have cross-reacting antibodies that lead to positive Lyme multiplex results. This could be because of a lack of test specificity or a result of molecular mimicry. Horses with false positive CSF and CSF : serum ratio results had a number of different postmortem diagnoses including EPM, degenerative myelopathy, cervical vertebral stenotic myelopathy, lymphoma, bacterial meningitis, and granulomatous meningoencephalitis. One final possibility is that some horses may have \textit{Borrelia} infection of the CNS without histologic changes, which leads to the (accurate) identification of intrathecal antibody production despite lack of histologic evidence for neuroborreliosis. Central nervous system infection without disease is considered plausible; 1 pony in an experimental infection study had \textit{B. burgdorferi} identified in the meninges by PCR without accompanying histologic lesions.\textsuperscript{19} \textit{Borrelia burgdorferi} does not produce toxins or extracellular matrix-degrading proteases, and clinical disease results from the host immune response generating inflammation.\textsuperscript{14} \textit{Borrelia burgdorferi} undergoes extensive antigenic variation to evade the host immune response, and successful evasion leads to infection without disease, as seen in wildlife reservoirs such as mice, which have no pathology despite lifelong infection.

Although horses with neuroborreliosis could not be reliably differentiated from horses without the disease on the basis of Lyme multiplex results, a few interesting findings warrant mention. None of the horses with neuroborreliosis were serologically positive for OspC antibodies, which might indicate that neuroborreliosis is a later manifestation of Lyme disease in horses, occurring more than 4–5 months after infection. Despite not having received Lyme vaccines, 7/90 (8%) horses (1 with neuroborreliosis and 6 with other neurologic diseases) were serologically positive for OspA antibodies. This proportion is similar to that documented in southwest Virginia, where 6% of healthy, nonvaccinated horses were seropositive for OspA antibodies.\textsuperscript{2} The magnitude of OspA antibody response in American human patients with Lyme arthritis has been directly correlated with more severe and prolonged disease.\textsuperscript{20,21} In our study, more horses with positive CSF OspA results or positive CSF : serum OspA ratios had a final diagnosis of another neurologic disease than neuroborreliosis. Therefore, the presence of OspA antibodies cannot be directly linked to more severe or chronic disease in horses.

Given the results of our study, simultaneous testing of serum and CSF to calculate a CSF : serum ratio using the Lyme multiplex is not a reliable means of identifying equine neuroborreliosis cases. Sensitivity was very poor (40%) and specificity was fair (79%), yielding a very low positive predictive value (19%) but a relatively high negative predictive value (91%). This conclusion does not mean that the assay is not useful, only that its results should be interpreted cautiously in light of other clinical information about the horse. Based on our experience and other expert opinion, several diagnostic criteria should be utilized for a presumptive (antemortem) diagnosis of neuroborreliosis in the horse. First, the horse should have had possible exposure to \textit{Borrelia} by residence in or travel to an endemic area. Second, the horse should show neurologic signs for which other potential etiologies have been excluded by appropriate diagnostic testing. There are no pathognomonic signs for neuroborreliosis; previous cases have shown a variety of clinical signs that potentially mimic diseases including EPM, West Nile virus, cervical vertebral stenotic myelopathy, and botulism. However, many affected horses have clinical signs that are most consistent with multifocal or diffuse nervous system involvement, often with cranial nerve involvement and often suggestive of meningitis, polyradiculoneuritis, or polyneuritis. These clinical signs should increase suspicion of neuroborreliosis, although negative diagnostic test results for other potential causes of disease remain imperative. Third, abnormal CSF results, particularly those indicative of meningitis, with either a neutrophilic or lymphocytic pleocytosis, should increase suspicion for the disease. Finally, some horses with neuroborreliosis might have additional supportive evidence of nervous system infection, with either positive CSF PCR results or immunological test results suggestive of intrathecal antibody production. However, it is important to recognize that...
negative Lyme multiplex assay results do not exclude neuroborreliosis, and positive results do not confirm it.

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Authors declare no conflict of interest.

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
Amy L. Johnson http://orcid.org/0000-0003-2507-0040

REFERENCES

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Signalment and Lyme multiplex assay results for 10 horses with a postmortem diagnosis of neuroborreliosis.
Table S2. Signalment, necropsy diagnosis, and Lyme multiplex assay results for 70 horses with neurologic disease other than neuroborreliosis.
Table S3. Signalment, postmortem diagnosis, and Lyme multiplex assay results for 10 normal horses.

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