A New Sensitive Lyme Multiplex Assay to Confirm Neuroborreliosis in Horses: A Case Report

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Neuroborreliosis is still difficult to diagnose in horses. Clinical signs of neurologic disease in horses housed in an endemic area may increase suspicion, but the diagnosis requires confirmation by detection of antibodies to Borrelia burgdorferi or pathogen in the cerebrospinal fluid (CSF). Antibody detection is often difficult because of the lack of assays with the required analytical sensitivity to measure the relatively low antibody concentrations in CSF. Serum antibodies to B. burgdorferi on their own are not sufficient to diagnose neuroborreliosis because serum titers are observed frequently in healthy horses that are kept in endemic areas. Polymerase chain reaction (PCR) analysis of CSF from affected horses for B. burgdorferi supports the diagnosis if positive but often fails to detect the pathogen because of low concentrations or inconsistent occurrence of the pathogen in CSF. A new fluorescent bead-based assay with a greater analytical sensitivity than existing diagnostic tests has been developed to detect antibodies to B. burgdorferi. This assay results in the reliable detection of much lower antibody concentrations in both serum and CSF and can be used to aid in the diagnosis of neuroborreliosis as demonstrated in this report by the clinical case of a horse. Authors’ addresses: Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center (Wagner and Glaser) and Department of Clinical Sciences (Divers), College of Veterinary Medicine, Cornell University, Ithaca, NY 14853; New England Equine Medical and Surgical Center, Dover, NH 03820 (Bartol, Mahar); and the Department of Clinical Studies, New Bolton Center, University of Pennsylvania, Kennett Square, PA 19348 (Johnson); e-mail: bw73@cornell.edu. *Corresponding author. © 2011 AAEP.

1. Introduction

Lyme disease is a zoonotic, vector-borne disease transmitted by infected ticks (Ixodes). In Europe and Asia, the disease commonly is caused by the spirochetes Borrelia garinii and Borrelia afzelii, whereas in the United States, Borrelia burgdorferi sensu stricto strains are present.1,2 The multisystemic disease occurs worldwide and has been reported in humans, dogs, horses, and other mammalian species. Clinical signs described in horses include chronic weight loss, sporadic lameness, low-grade fever, muscle tenderness, chronic-
cally poor performance, swollen joints, arthritis, and diverse orthopedic problems. In addition, neurological signs such as depression, behavioral changes, dysphagia, head tilt, and encephalitis were reported in chronic cases. Nevertheless, the diagnosis of neuroborreliosis in a horse is often difficult to confirm because similar clinical signs of neurological diseases can be induced by other equine pathogens.

In general, the diagnosis of Lyme disease in horses is made on the basis of the horse being housed in an endemic area, ruling out other causes of clinical signs and a high antibody titer to *B. burgdorferi*. Diagnostic antibody testing is usually performed by ELISA followed by Western blotting. This procedure is still considered the gold standard for Lyme disease testing and is recommended by the Center of Disease Control (CDC) for testing of human serum samples. ELISA and Western blot testing for Lyme disease has been established for samples from horses and is performed by several diagnostic laboratories. Experimental infection of ponies with *B. burgdorferi* by infected ticks confirmed that all exposed horses seroconverted a few weeks after infection. Although antibody testing works generally well for serum samples, it often fails for cerebrospinal fluid (CSF) samples, probably because conventional diagnostic assays for Lyme testing might not be sensitive enough to detect the relatively low antibody concentrations in the CSF. Low antibody concentrations in CSF in response to central nervous system (CNS) infection cause a difficulty in diagnosing neuroborreliosis because the confirmation of CNS infection requires the identification of antibodies or agent in neurological tissue or CSF.

New testing strategies and technologies are needed to improve the differential diagnosis of neurological conditions, including neuroborreliosis in horses. The Animal Health Diagnostic Center at Cornell University has developed a new multiplex assay for the diagnosis of Lyme disease in dogs and horses. Multiplex assays use the principle of simultaneous detection of soluble analytes in biological samples. They are based on fluorescent beads coupled with individual antigens that provide the matrix of the assay. The Lyme multiplex assay uses different outer surface proteins (Osp) of *B. burgdorferi*, such as OspA, OspC, and OspF, as markers for vaccination and/or early or chronic infection. Multiplex assays typically detect antibodies in the pg/mL range, whereas ELISA detects ng/mL and Western blotting detects μg/mL. Thus, the multiplex assay probably is advantageous in situations when antibody concentrations are low, such as early after infection or in CSF samples of most horses with neurological signs. We used a combined diagnostic approach of all three antibody tests together with polymerase chain reaction (PCR) analysis on the CSF sample of a horse with a neurologic condition to confirm neuroborreliosis.

2. Materials and Methods

Detection of Antibodies to *B. burgdorferi*

Antibodies against *B. burgdorferi* were measured in serum and CSF of a horse with neurological signs using the kinetic ELISA and Western blotting procedure for horse samples performed at the Animal Health Diagnostic Center at Cornell University. ELISA and Western blotting used a whole *B. burgdorferi* cell lysate for antibody detection.

Both samples, serum and CSF, were also tested by multiplex analysis as previously described. In brief, recombinant *B. burgdorferi* OspA, OspC, and OspF antigens were expressed in *Escherichia coli* and coupled to fluorescent beads. OspA was coupled to bead 33, OspC to bead 34, and OspF to bead 37. The coupling was performed according to the recommended protocol from the bead supplier. For the multiplex assay, beads coupled with OspA, OspC, and OspF were sonicated, mixed, and diluted in blocking buffer (PBS with 1% [w/v] BSA and 0.05% [w/v] sodium azide) to a final concentration of 1 × 10^5 beads/mL each and 5 × 10^5 beads/each were used per microtiter well. Equine serum was diluted at 1:400 in blocking buffer and CSF at 1:2. Previously tested negative, low positive and high positive equine sera and beads incubated with blocking buffer alone were run as positive and negative controls on each assay plate. Millipore Multiscreen HTS plates were soaked with PBS-Tween using an ELx50 plate washer for 2 minutes. The solution was aspirated, and 50 μL of each control serum or equine sample was applied to the plates. Then, 50 μL of bead solution was added to each well and incubated for 30 minutes on a shaker at room temperature. The plate was washed with PBS-Tween and 50 μL of a biotinylated goat anti-horse IgG (H + L) antibody diluted 1:3000 was used for detection and incubated for 30 minutes as above. After washing, 50 μL of streptavidin-phycocerythrin diluted 1:100 in blocking buffer was added. Plates were incubated for 30 minutes as above and washed. The beads were resuspended in 100 μL of blocking buffer and each plate was placed on the shaker for 15 minutes to resuspend the beads. The assay was analyzed in a Luminex 200 instrument. The data were reported as median fluorescent intensities.

PCR for *B. burgdorferi*

PCR was performed at the Animal Health Diagnostic Center at Cornell University. DNA was extracted from 200 μL of CSF, using the DNeasy Blood and Tissue extraction kit, following the manufacturer’s suggested protocol. DNA was amplified using the G1 primer pair targeting OspA. PCR products were visualized after electrophoresis in a 2% agarose gel by ethidium bromide staining and UV illumination. The extracted DNA from the CSF sample was also tested in triplicate using a real time 5′ nuclease assay targeting the flagellin gene.
3. Results

Clinical Case

A 14-year-old Thoroughbred gelding used as a hunter was examined at New England Equine Medical and Surgical Center in New Hampshire because of progressive weakness over a 6-month period. He had previously received intra-articular medication for suspected hock osteoarthritis with little improvement. On hospital presentation, the horse was thin (body condition score, 3.5) and had moderate to severe proprioceptive deficits in both hind limbs. The gelding was also judged to be moderately lame in the left fore and left hind limbs. Manual palpation and manipulation of the back and neck suggested that the horse was painful in the back and had an abnormally limited range of motion in his neck. The horse also seemed painful on manipulation of the tail, and coccygeal vertebrae 1 and 2 appeared to be in a depressed position in comparison to the last sacral vertebrae and the rest of the coccygeal vertebrae. A bone scan was performed, which revealed increased radionucleotide uptake in the left front medial sesamoid bone, right lower carpus and both hocks, distal femurs, and base of the tail. Radiographs revealed erosion of the left front medial sesamoid bone, bilateral lower hock, and right front lower carpus osteoarthritis. Ultrasound evaluation of the left front fetlock showed that the boney erosion was under the intersesamoidal ligament. Cervical radiographs showed mild to moderate osteoarthritic of the dorsal aspect of both C5-C6 and C6-C7. A myelogram performed under general anesthesia did not show any evidence of spinal cord compression. A sample of CSF collected from the cisterna magna at the time of the myelogram was submitted for routine cytoclogic evaluation, equine protozoal myelitis, and Lyme testing. The CSF had an abnormal yellow appearance with a lymphocytic pleocytosis of 410 WBC/μL (normal <5/μL) characterized by a majority of small lymphocytes with scattered reactive and granular lymphocytes. The CSF protein was 148 mg/dL (normal <90mg/dL), and there were minimal (n = 3) RBCs in the fluid. The cytologic interpretation was either a lymphocytic inflammatory disease or spinal lymphoma. Differential diagnoses for the disease based on all of the information at that time included lymphosarcoma, neuroborreliosis, polynueuriitis equi, and parasitic spinal cord disease in addition to osteoarthritis in several areas of the body. Thoracic and abdominal ultrasound were performed trying to identify any other evidence of disease. Thoracic ultrasound was within normal limits and an enlarged spleen was identified on abdominal ultrasound. At the owners request, the horse was also tested for polysaccharide storage myopathy (PSSM) by muscle biopsy of the semimembranosus muscle. Results were consistent with moderate PSSM and myogenic atrophy. The genetic test for Type I PSSM (GYSI) was N/N. The serum and CSF were negative on an immunofluorescence assay for equine protozoal myelencephalitis EPM. Flow cytometric analysis of cells in the CSF showed that 58% of the lymphocytes were CD19+ B-cells and 22% were T-cells, including both CD4+ T-helper cells and CD8+ cytotoxic T-cells, which could have been compatible with lymphoma and reactive lymphocytes or lymphocytic inflammatory disease.

The horse was treated with corticosteroid injections of cervical facets of C5–6 and C6–7, as well as at the tail luxation. One dose of tiludronate was administered IV on 2/7. The horse was treated with doxycycline (10 mg/kg PO q 12 hours since 2/13) and phenylbutazone (1 g PO SID from 2/13 until 3/5) and was kept on a low carbohydrate and high fat (9%) diet. According to the owner and trainer, the horse improved (by 3/14) and was described as stronger and more energetic, with mild hind limb ataxia.

Diagnostic Testing to Support Neuroborreliosis

Serum and CSF samples were analyzed by conventional ELISA and Western blotting procedures for antibodies to *B. burgdorferi*. The ELISA value for serum was 110, which falls into the negative interpretative range of the assay; the ELISA value for the CSF was 158, which is considered equivocal. An equivocal result can originate from very low antibody concentrations or nonspecific reaction of a sample and is thus inconclusive. The confirmative Western blot result for both samples was negative. Overall, ELISA and Western blot testing provided no indication for exposure to *B. burgdorferi* or neuroborreliosis in this horse.

The serum and CSF samples were then tested by multiplex analysis because of its higher analytical sensitivity and ability to detect lower concentrations of *B. burgdorferi*--specific antibodies. The results are shown in Table 1. The multiplex assay was positive for antibodies to OspA in the CSF and OspC in serum and CSF, whereas the result for antibodies to OspF was negative in both samples. The sample run was repeated on another day and revealed the same result. Because of the clinical signs, the fact that the antibodies were found in the CSF, as well as diagnostic test results that excluded other likely

### Table 1. Antibodies to *Borrelia burgdorferi* OspA, OspC, and OspF Detected by Multiple Analysis

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Antibodies to†‡</th>
<th>OspA</th>
<th>OspC</th>
<th>OspF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1:400</td>
<td>1658</td>
<td>1997</td>
<td>408</td>
</tr>
<tr>
<td>CSF</td>
<td>1:2</td>
<td>4554</td>
<td>2542</td>
<td>312</td>
</tr>
<tr>
<td>Positive if†‡</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>&gt;1250</td>
<td></td>
</tr>
</tbody>
</table>

* Values represent median fluorescent intensities (MFI).
† The upper detection range of the assay is at around 20,000 MFI and is indicative of very high antibody concentrations.
‡ Positive values.
diseases, we concluded that this horse may be affected with neuroborreliosis.

To confirm the serological result, a PCR analysis of the CSF was performed. A faint band was visualized by qualitative PCR at the expected size of 540 base pairs for the DNA from the CSF sample (data not shown). The sample was subsequently tested by real-time PCR. Two of the three replicates tested positive with cycle threshold values of 34.8 and 34.07 (Table 2). Both amplification assays targeting different genes identified *B. burgdorferi* and results suggested that the agent was present in relatively low copy number, with detection of the target sequences near the limit of analytical sensitivity for both assays. The 5' nuclease assay has a limit of detection of 5 copies per reaction and the observed cycle threshold values from the CSF sample were consistent with the presence of 5 to 10 copies of the target sequence in the reaction. This could be extrapolated to an approximate original concentration of 500 to 1000 spirochetes/mL of CSF.

4. Discussion

Neurologic disease induced by *B. burgdorferi* in any species is difficult to diagnose. Clinical signs of progressive ataxia and weakness can be similarly induced in horses by various equine pathogens including West Nile virus, Western and Eastern encephalitis virus, *Sarcocystis neurona*, Equine herpesvirus type 1, and others. All general considerations for diagnosing Lyme disease also apply to neuroborreliosis; the diagnosis is based on the horse being housed in an endemic area, compatible clinical signs, ruling out other causes for the clinical signs and a serological titer confirming exposure to *B. burgdorferi*. Antibodies to *B. burgdorferi* or a positive PCR result for the pathogen in the CSF are additional confirmatory tests supporting a diagnosis of neuroborreliosis.

Nevertheless, neuroborreliosis is yet rarely diagnosed and described by only a few reports. The difficulties in confirming neuroborreliosis may be partially due to a lack of sensitivity of the conventional antibody tests, which could fail to detect the immune response to *B. burgdorferi* in CSF samples. This is supported by the data collected from CSF samples submitted from horses with neurological signs for immunological Lyme testing to the Animal Health Diagnostic Center at Cornell University. A total of 38 paired serum and CSF samples from horses with neurological signs were submitted during the past two years, 29 of which were negative in serum and CSF by ELISA and Western blot testing and nine horses had serum antibodies indicative for exposure to *B. burgdorferi*. However, the nine corresponding CSF samples were negative for Lyme testing by ELISA and confirmatory Western blotting, which suggests that the horses had a neurologic disease other than neuroborreliosis or that the pathogen was of relatively low concentration in CSF, causing low or undetectable local antibody concentrations, even if neurological signs are obvious or severe.

Serologic tests of improved analytical sensitivity compared to conventional ELISA and Western blot assays may overcome the difficulties in confirming neuroborreliosis. For the neurological case of the horse described here, we showed that a new fluorescent bead-based multiplex assay detected antibodies to *B. burgdorferi* in the CSF and serum samples despite the failure of the conventional assays to identify the horse's immune response. The finding was supported by qualitative and real-time PCR analysis both indicating low copy numbers of *B. burgdorferi* in the CSF of the horse. Although PCR yielded positive results in this case, in human medicine, PCR from CSF samples is generally low-yield in the diagnosis of neuroborreliosis. Therefore, diagnostic criteria for human neuroborreliosis generally do not include PCR testing but do include identification of *B. burgdorferi*-specific antibodies in CSF.

The increased analytical sensitivity of multiplex analysis compared to ELISA was described previously for other assays. Based on the finding presented here, the new Lyme multiplex for horses provides an improved diagnostic tool for the detection of antibodies to *B. burgdorferi* in biological samples including CSF. The Lyme multiplex assay, similar to a Western blot, can distinguish between antibodies to infection or vaccination by using various antigens of *B. burgdorferi*. The principle of this assay to use OspA, OspC and OspF as markers for vaccination (OspA) and infection (OspC and OspF) is based to the differential expression of these antigens on the surface of *B. burgdorferi*. The expression of individual antigens on the spirochete differs, depending on the environment. In the tick gut or in culture, *B. burgdorferi* expresses OspA, whereas OspC is expressed after the tick bite during transmission of the spirochetes and initial infection of the mammalian host, whereas OspF becomes expressed in the mammalian host and antibodies remain detectable during chronic infection. These differential expression patterns of

### Table 2. Cycle Threshold Values for the 5' Nuclease Assay Targeting the Flagellin Gene of *Borrelia burgdorferi*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Copy Numbers</th>
<th>Cycle Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF 1</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>CSF 2</td>
<td>34.84</td>
<td></td>
</tr>
<tr>
<td>CSF 3</td>
<td>34.07</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>28.82 (28.85 ± 1.28)</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>23.36 (25.62 ± 1.46)</td>
</tr>
<tr>
<td>PAC</td>
<td>50,000</td>
<td>21.74 (21.56 ± 0.28)</td>
</tr>
</tbody>
</table>

NAC, negative amplification control; PAC, positive amplification control.

Values in parentheses represent the expected values for the copy number controls.
the *B. burgdorferi* Osp antigens result in a variation of the onset of antibody responses after infection of mammals. Antibodies to OspC develop early after infection and are known as early infection markers in human patients. Antibodies to OspF are induced later during infection and seem to be maintained throughout the chronic infection stage. Although experimental studies are still missing, it is likely that similar antibody kinetics to the OspC and OspF antigens exist generally in mammals including horses. The antibody detection patterns observed in the horse described here were consistent with an early infection stage of Lyme disease because antibodies to OspC were present in serum and CSF, whereas those to OspF were not yet developed. This suggested that infection occurred within a few months of sample collection. In addition, increased antibodies to OspA were measured in the CSF. This phenomenon was observed previously in serum of exposed horses and can be seen in this species frequently after exposure to *B. burgdorferi* and in the absence of vaccination. Similarly, antibodies to OspA were measured in human patients with chronic Lyme disease. This suggests that the expression of OspA is not strictly associated with the survival of the spirochetes in the tick gut and can also occur under certain conditions in at least some mammalian hosts including horses.

Another benefit of utilizing the quantitative multiplex assay described here is the ability to calculate specific antibody indices. Antibodies are partitioned between the blood and CSF at a constant ratio, based on the restriction coefficient of the blood-brain barrier. Infection of the central nervous system induces intrathecal antibody production, which changes the ratio. Evaluation of specific antibody ratios has proven to be useful and more specific than existing tests in the diagnosis of equine protozoal myeloencephalitis. Relying solely on the presence of CSF antibodies in the diagnosis of equine neuroborreliosis is likely to result in false positive results due to normal diffusion of antibodies into CSF. By quantitatively evaluating paired serum and CSF samples, as performed in this case, the magnitude of intrathecal antibody production can be assessed. Although additional research is required to optimize test performance, use of this new multiplex assay to calculate a *B. burgdorferi*-specific antibody index will allow clinicians to more accurately diagnose horses with neuroborreliosis.

In summary, serological assays of improved analytical sensitivity can detect antibodies to *B. burgdorferi* in CSF samples and are likely to support the diagnosis of neuroborreliosis in horses.

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**References and Footnotes**


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