

Serologic Diagnosis of Equine Borreliosis: Evaluation of an In-Clinic Enzyme-Linked Immunosorbent Assay (SNAP[®] 4Dx[®])

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ABSTRACT

SNAP[®] 4Dx[®] (IDEXX Laboratories, Westbrook, ME) is a commercially available in-office test kit for the simultaneous detection of *Dirofilaria immitis* antigen and antibodies to *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia canis* in blood, plasma, or serum of dogs. The test kit is an enzyme-linked immunosorbent assay that uses the synthetic peptide C₆ derived from the IR6 region within the *Borrelia* membrane protein VlsE, peptides derived from the immunodominant p44 protein of *A phagocytophilum*, and the p30 and p30-1 protein of *E canis*. Studies with canine samples suggest that SNAP[®] 4Dx[®] is particularly useful in endemic areas because it can be conveniently and reliably used in the clinic to determine the infection status of a dog. We evaluated the performance of SNAP[®] 4Dx[®] for the detection of antibodies to Lyme and *Anaplasma* in equine serum samples from Northeastern United States and Alaska. A total of 164 equine samples obtained from the University of Connecticut were tested by SNAP[®] 4Dx[®], Lyme disease

immunofluorescence assay (IFA; VMRD, Inc., Pullman, WA) and QualiCode[™] *B burgdorferi* IgG/IgM Western Blot Kits (Immunetics, Cambridge, MA). For *A phagocytophilum*, an IFA was utilized. Of the serum samples tested, 109 were positive for Lyme and 54 samples were positive for *A phagocytophilum* by SNAP[®] 4Dx[®]. Twenty-four samples were co-infected with Lyme and *A phagocytophilum*. Of the 109 samples that tested positive for Lyme by SNAP[®] 4Dx[®], 106 were positive by the Lyme Western blot assay. The 3 discordant samples were positive by IFA and had a low reciprocal antibody titer of 64. All 54 samples that were positive for *A phagocytophilum* by SNAP[®] 4Dx[®] tested positive by IFA. Each of the SNAP[®] 4Dx[®]-negative samples was negative by Western blot and IFA. Thus, relative to Lyme Western Blot assay, SNAP[®] 4Dx[®] had a sensitivity and specificity of 100% and 95%, respectively, for the detection of antibodies to Lyme. Relative to IFA, SNAP[®] 4Dx[®] had a sensitivity and specificity of 100% for the detection of antibodies to *A phagocytophilum*. These results indicate that SNAP[®] 4Dx[®] can be successfully used to detect antibodies to *B burgdorferi* and *A phagocytophilum* in infected horses.

INTRODUCTION

Lyme disease caused by *Borrelia burgdorferi* is widespread in the Northeastern and mid-Atlantic United States, Minnesota, Wisconsin, and Northern California. *Borrelia burgdorferi* is maintained in a 2-year enzootic cycle that involves *Ixodes* spp ticks and mammals. Infection generally occurs from larval, nymph, or adult tick feeding. In horses, it is not known if larval and nymph bites play an important role in Lyme infection. Ticks are usually attached for at least 24 hours for *B burgdorferi* transmission.¹

Horses from endemic areas may have serologic evidence of exposure. Seroprevalence rates in horses ranged from 6% to 35% or higher.^{2,3} More recent studies by Magnarelli et al⁴ indicated that nearly 50% of adult horses in some areas of the Northeastern United States are infected or have been infected. Prevalence in horses in other parts of the United States has not been reported but would be expected to mimic human and canine infection rates. Seroprevalence in horses might be expected to be higher than humans because of the increased risk of ticks attaching to horses for greater than 24 hours.

Clinical signs most commonly attributed to Lyme disease in horses include low-grade fever, stiffness and lameness in more than one limb, muscle tenderness, hyperesthesia, swollen joints (rarely), and behavioral changes.^{4,5} In one report, *Borrelia* infection (confirmed by serology or spirochetemia) was more common in horses with lameness and/or behavioral changes than in horses in the same region without those clinical signs.^{5,6}

Granulocytic anaplasmosis is caused by *Anaplasma phagocytophilum* and is endemic in the Northeastern and Midwestern United States and Northern California. It is transmitted by both *Ixodes scapularis* and *Ixodes pacificus*.⁷ Granulocytic ehrlichiosis in horses is a seasonal disease (late fall to spring), reflecting the activity of the tick vectors at that time. This disease in the horse is characterized by fever, lethargy, stock-

ing edema, thrombocytopenia, leukopenia, anemia, and petechiae.^{7,8} Current diagnostic tests are based on immunofluorescence assay (IFA) serology or polymerase chain reaction for acute cases.⁴

The purpose of our study was to compare the performance of a commercially available SNAP® 4Dx® for the detection of antibodies to *B burgdorferi* and *A phagocytophilum* using sera from infected and uninfected horses.

MATERIALS AND METHODS

Equine Samples

Lyme positive equine serum samples were purchased from Connecticut Veterinary Diagnostic Laboratory (CVDL), Department of Pathobiology, College of Agriculture & Natural Resources University of Connecticut Storrs, CT. These samples were submitted to CVDL for Lyme Western blot (LWB) testing. Blood, plasma, and serum from Lyme-vaccinated (n = 6) and -infected horses (n = 3) were obtained from Rhinebeck Equine LLC, Rhinebeck, NY. The vaccinated horses received 2 doses of canine LymeVax® at 6-month intervals (Fort Dodge Laboratories, Inc., Fort Dodge, IA).

Diagnostic Testing

Lyme Western Blots

Lyme Western blot analysis was performed using the QualiCode™ *Borrelia burgdorferi* IgG/IgM Western Blot Kits (Immunetics, Inc., Boston, MA). The Western blot analyses were performed as per manufacturer's instructions. The assay was optimized with equine serum samples. A checkerboard titration was performed using Lyme-positive and -negative serum samples obtained from non-endemic area for Lyme (Alaska) (n = 10) and different dilutions of goat anti-horse IgG conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). A serum dilution of 1:50 and conjugate dilution of 1:750 was found to be optimal and was used to test clinical samples. Specific bands obtained were identified by comparison of the results obtained with the positive control supplied with the kit.

SNAP® 4Dx®

The SNAP® 4Dx® Test Kit is an enzyme immunoassay for the simultaneous detection of *Dirofilaria immitis* antigen, antibody to *B burgdorferi*, *E canis*, and *A phagocytophilum* in canine whole blood, serum, or plasma (Figure 1). The assay was run as per manufacturer's instructions. Briefly, 3 drops of sample (whole blood, serum, or plasma) was added into a sample tube followed by the addition of 4 drops of conjugate. The sample tube was mixed thoroughly by inverting the tube 3-5 times and the entire contents of the sample tube were added to the SNAP® device. The test results were read at 8 minutes after device activation. Since SNAP® test uses antigen-specific conjugates, the test can work with samples derived from multiple species.

Immunofluorescence Assay

Lyme disease IFA was performed using commercially available IFA slides that were purchased from VMRD, Inc., Pullman, WA. The IFA was optimized using equine samples. Distinct fluorescence of spirochetes at

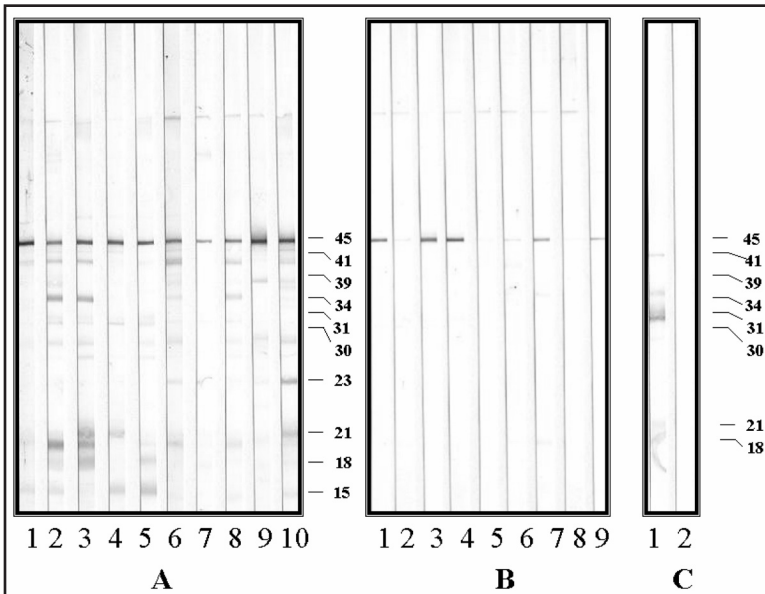
dilutions $\geq 1:64$ was considered evidence of *B burgdorferi* exposure. Anaplasmosis IFA was performed using slides available from Protatek International, Minneapolis, MN. Distinct fluorescence at a sample dilution of $\geq 1:80$ was considered evidence for exposure to *A phagocytophilum*.

RESULTS

Lyme Western Blot Analysis

Lyme-infected horse sera and sera obtained from Alaskan horses (tested negative by Lyme IFA) were used to analyze the banding pattern obtained with Lyme-infected equine sera using the QualiCode™ *Borrelia burgdorferi* IgG/IgM Western Blot kit. Figure 1 depicts the immunoblot profile of sera from Lyme-infected horses and negative controls. Lyme-infected equine serum samples reacted to several major *B burgdorferi* proteins. These correspond to bands at p45, 41, 39, 34, 31, 30, 23, 21, 18, and 15 kDa. Several Lyme-negative samples reacted to the 45-kDa band, but did not react to the low molecular weight diagnostic bands.

Figure 1. Representative results of the IgG/IgM Western blot analysis of equine serum samples to determine total antibody to whole-cell *B. burgdorferi*. Molecular masses for key bands are indicated in kilodaltons. Panel A: Lanes 1-10 were developed with Lyme-positive equine serum samples. Panel B: Lanes 1-9 were developed with Lyme-negative equine serum samples. Panel C: Lane 1, Kit-positive control; Lane 2, Kit-negative control.



The diagnostic interpretation of LWB as per manufacturer's instructions is as follows: ≥ 2 bands in the p0-p14 region without both p31 and p34 = positive; < 2 bands in the p30-p4 region = negative; and ≥ 2 bands in the p30-p14 region = vaccinated.

One hundred sixty-four equine samples were tested by SNAP® 4Dx® and by LWB. One-hundred six of 164 samples were positive by LWB. Six serum samples from vac-

cinated horses were also tested by LWB. All 6 samples reacted strongly to the vaccine bands at 31 and 34 kDa (OspA and OspB, respectively; Figure 2). In addition, they bound to bands corresponding to molecular masses of 18, 21, and 23 kDa.

Figure 2. Lyme Western blot analysis of serum samples from vaccinated horses. Molecular masses for key bands are indicated in kilodaltons. Panel A: Lanes 1-6, vaccinated horse samples. Panel B: LWB controls. Lane 1, Lyme-positive equine serum sample; Lane 2, Negative sample; Lane 3, Kit-positive control; Lane 4, Kit-negative control.

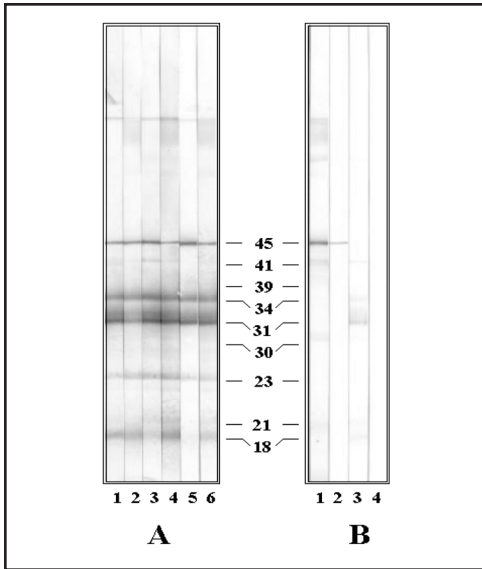
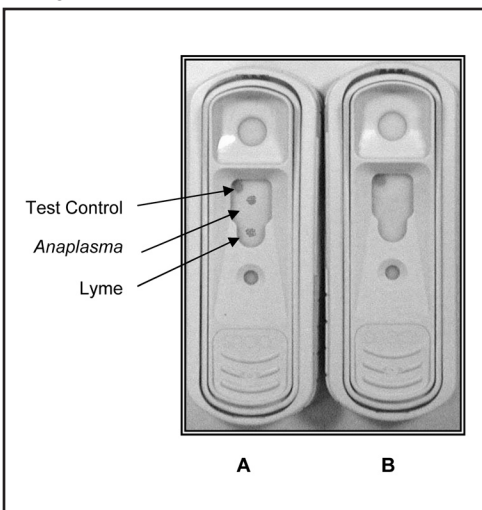


Figure 3. Representative results of the SNAP® 4Dx® assay. A. Lyme- and *Anaplasma*-positive sample. B. Negative sample.



SNAP® 4Dx®

Whole blood, plasma, or serum from Lyme-infected horses performed similarly when tested in the C₆-based SNAP® 4Dx® assay. A set of representative devices tested with both Lyme- and *Anaplasma*-positive and -negative samples are shown in Figure 3. One-hundred nine of 164 equine tested positive by the SNAP® 4Dx® assay. However, only 106 of 109 SNAP® 4Dx®-positive samples were LWB positive. Relative to LWB, sensitivity and specificity of SNAP® 4Dx® was 100% and 95%, respectively (Table 1). The 3 LWB-negative samples were tested by IFA and were weakly positive with a low reciprocal antibody titer of 64. Of the 109 Lyme-positive samples, 24 were co-infected with *A phagocytophilum* (Table 1).

Five of the 6 samples from horses vaccinated with the Lyme vaccine tested negative for antibodies to the C₆ analyte in SNAP® 4Dx®. One sample that was positive for antibodies to Lyme by SNAP® 4Dx® was infected prior to vaccination (J. Williams, Personal communication).

Fifty four of the 164 samples tested positive by SNAP® 4Dx® for antibodies to *A phagocytophilum*. All 54 samples were positive by IFA (Table 2). Each of the 110 equine samples that were negative by the SNAP® 4Dx® was also negative by IFA. Thus, for the sample population tested in this study, SNAP® 4Dx® had a sensitivity and specificity of 100% compared to IFA for the detection of antibodies to *A phagocytophilum*. Of the 54 samples that tested positive for *A phagocytophilum*, 24 were co-infected with *B burgdorferi* (Table 2).

DISCUSSION

This study evaluated the performance of the in-clinic SNAP® 4Dx® assay for the detection of antibodies specific to *B burgdorferi* and *A phagocytophilum* in infected horses. The test kit is an enzyme-linked immunosorbent assay (ELISA) that uses a synthetic peptide (C₆) derived from the IR6 region within the *Borrelia* membrane protein VlsE⁹ and a peptide derived from the p44 gene product of *A phagocytophilum*. Studies with

Table 1. Performance of SNAP® 4Dx® compared with LWB.

		SNAP® Dx®	
		+	-
Qualicode™ Lyme Western Blot	+	106 ^a (1V) ^b	0 (5V) ^c
	-	3*	55
Relative to Western Blot			
% Specificity	100		
% Sensitivity	95		
*Positive by IFA			

^a24 samples were co-infected by *Anaplasma*

^bLyme vaccinated samples

^cPrior infection and vaccinated later

Table 2. Performance of SNAP® 4Dx® compared with IFA.

		SNAP® Dx®	
		+	-
Qualicode™ Lyme Western Blot	+	54 ^a	0
	-	0	110
Relative to Western Blot			
% Specificity	100		
% Sensitivity	95		
*Positive by IFA			

^a24 samples were co-infected by Lyme

canine samples suggests that SNAP® 4Dx® is particularly useful in Lyme-endemic areas because it can be conveniently and reliably used in the clinic to determine the infection status of a dog irrespective of its vaccination history.¹⁰ Studies with experimentally infected dogs demonstrated a strong humoral response to the C₆ peptide 4-5 weeks after infection with tick-borne *B burgdorferi*.⁹ In another study, SNAP® 4Dx® was shown to be highly specific (99.6%) and sensitive (94.4%) for detection of Lyme-positive dogs.¹¹ In the present study, the SNAP® 4Dx® performed well with equine samples with a high sensitivity of 100% and a specificity of 95% compared to LWB.

Suspected clinical cases of Lyme disease in horses have been recognized in endemic areas. The clinical signs of equine Lyme disease can be varied, and some of the signs that have been documented include chronic

weight loss, sporadic lameness, and laminitis.⁶ Proper diagnosis of Lyme disease depends on several factors. Unfortunately, Lyme disease is often difficult to diagnose in horses because its symptoms and signs mimic those of many other diseases. Thus, due to the nature of the disease, it is often overlooked or misdiagnosed. Diagnostic tests should be performed to rule out other diseases that cause similar signs before attributing clinical signs of Lyme disease.

Serologic testing is the only practical means of confirming *B burgdorferi* infection. There are a number of serologic test methods for detecting antibodies to *B burgdorferi*. These include IFA, ELISA, or Western blot and detect either total or class-specific antibodies (IgM or IgG). Currently, horses are tested for the presence of antibodies to Lyme by ELISA followed by Western blot confirmatory test. These ELISA assays produce a large number of false positives, resulting in many time-consuming confirmatory Western blot tests, which can delay reporting of results. The high specificity of the C₆ Lyme analyte in SNAP® 4Dx® minimizes false positives, thereby eliminating the majority of confirmatory Western blots.¹² In addition, because it was designed to be a rapid in-clinic assay, the results are read in 8 minutes after sample application and thus, the veterinarian can report the results to the client immediately after testing.

SNAP® 4Dx® detects antibody to C₆ that is only expressed during natural *B burgdorferi* infection and antibodies to C₆ peptide do not cross react to OspA.⁹ Hence, the Lyme

analyte in SNAP® 4Dx® can distinguish between the response to natural infection and the response to vaccination. We tested samples from horses vaccinated with LymeVax®. Five of the 6 horses that were vaccinated were negative by SNAP® 4Dx®. One horse that was positive by SNAP® 4Dx® tested positive by IFA and Western blot positive before vaccination indicating that the horse was previously exposed to *B burgdorferi*. Additional field studies are needed to validate the use of SNAP® 4Dx® in large number of vaccinated horses.

The current diagnosis of *A phagocytophilum* infection in horses is based on clinical signs, combined with laboratory findings. In addition, serological testing of antibodies to *A phagocytophilum* is performed using indirect IFA or whole-cell ELISA. False-positive test results can occur when whole-cell antigen-based ELISA or IFA are used. The SNAP® 4Dx® test has been developed to detect antibodies to *A phagocytophilum* in dogs. In the current study, SNAP® 4Dx® was able to detect antibodies in horses infected with *A phagocytophilum* with a sensitivity and specificity of 100% for the population of samples tested. Similar observations were made in a recent study that showed SNAP® 4Dx® test had a sensitivity and specificity of 99.1% and 100%, respectively, compared to IFA for canine samples.¹¹

The use of SNAP® 4Dx® allowed us to identify animals that are co-infected with both *A phagocytophilum* and *B burgdorferi*. Of the 164 samples tested in this study, 24 were dually infected. Dual infections in horses have been reported in dogs and horses, and one recent study indicates that co-infection in dogs can increase the risk of clinical signs.¹³ The observation of dual infections is not surprising since both organisms are transmitted by the *Ixodes* spp of ticks.

In conclusion, SNAP® 4Dx® can detect antibodies to *B burgdorferi* and *A phagocytophilum* in infected horses. None of the equine samples tested in the present study reacted with either *E canis* or the heartworm analytes in the SNAP® 4Dx® assay.

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