Performance of a Recombinant LipL32 Based Rapid In-clinic ELISA (SNAP® Lepto) for the Detection of Antibodies Against *Leptospira* in Dogs

Curtis, KM[1]

Foster, PC[1]

Smith, PS[1]

Monn, MP

Stillman, BA[1]

Chandrashekar R[1]

Lappin, MR[2]

Goldstein RE[3]

[1]*IDEXX Laboratories Inc, Westbrook, ME*

[2]*Colorado State University, Fort Collins, CO*

[3]*Animal Medical Center, New York, NY.*

KMC: kris-curtis@idexx.com
PCF: paulette-foster@idexx.com
PSS: phillip-smith@idexx.com
MPM: michael-monn@idexx.com
BAS: brett-stillman@idexx.com
RC: chandra@idexx.com
MRL: michael.lappin@colostate.edu
REG: Richard.Goldstein@amcny.org

Correspondence: K. Curtis, PhD. kris-curtis@idexx.com.

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ABSTRACT

The purpose of this study was to compare the performance of SNAP® Lepto, a LipL32-based rapid enzyme-linked immunosorbent assay (ELISA) to the serological gold standard microscopic agglutination test (MAT) for the detection of antibodies to *Leptospira* spp in a broad population of canine samples. Four hundred and sixty serum samples submitted for MAT testing at IDEXX Reference Laboratories were tested on SNAP Lepto. Positive percent agreement between the two tests was 83.2% for samples with peak MAT titers ≥ 1:800, 64.9% for samples with peak MAT titers ranging from 1:100 to 1:400, and 79.2% for samples with peak MAT titers ≥ 1:100. Negative percent agreement between these tests was 82.1% in this population. One hundred and fifty MAT negative serum samples from healthy dogs in Alaska were tested by SNAP Lepto, and the percent agreement was

96.0%. Fifty-two serum samples positive for anti-*Borrelia burgdorferi* antibodies were tested. All 52 samples were negative by MAT, and 51/52 samples were negative on SNAP Lepto. Serial serum samples from 28 dogs following *Leptospira* vaccination were tested by MAT and SNAP Lepto. MAT results were positive in 27/28 dogs at week 3 (highest peak titer of 1:3200), 28/28 dogs at week 4 (highest peak titer of 1:6400), 3/21 dogs at week 52 (highest peak titer of 1:200), and 15/19 dogs at week 56 (highest peak titer of 1:1600). In comparison, positive SNAP Lepto test results were observed in 15/28 dogs at week 3, 22/28 dogs at week 4, 5/21 dogs at week 52, and 16/19 dogs at week 56. In this study, SNAP Lepto demonstrated similar performance to the MAT for the detection of antibodies to *Leptospira* spp.

INTRODUCTION

Canine leptospirosis is a zoonotic bacterial infection with worldwide distribution that is caused by spirochetes of the genus *Leptospira*.[1-3] Infection in dogs most commonly occurs through direct contact with urine or water containing *Leptospira*. Clinical presentation in dogs is often variable and can range from subclinical infection to an acute, fatal disease that is characterized by multi-organ dysfunction. Currently, laboratory testing is required for proper differential diagnosis.

Because culture of the organism from blood or urine is difficult and not sensitive, serology is typically used for diagnosis of leptospirosis.[1,4-6] The microscopic agglutination test (MAT) for detection of antibodies is the most common diagnostic test used today to aid in the diagnosis of canine leptospirosis.[3,4,6] The MAT is performed by mixing serial dilutions of canine sera with cultured *Leptospira* organisms of different serovars representing different serogroups. The titer against a specific serogroup is defined as the highest dilution of the sera that caused 50% or more agglutination of the organisms representing that serogroup. The serogroup with the highest titer is typically interpreted

as the infecting serogroup; however, due to a high degree of cross-reactivity across serogroups, that may not always be accurate.[7] A peak MAT titer of > 1:3200 to a vaccinal serovar and > 1:1600 to a non-vaccinal serovar is suggestive of active infection.[4] Standardization of the MAT across laboratories has been difficult, and variations in titers reported may occur with multiple testing events on the same sample and also between different laboratories.[8]

LipL32 is the major outer membrane protein of *Leptospira*, with the highest number of copies per organism than any other membrane protein.[9,10] LipL32 is expressed only by pathogenic *Leptospira*, and is highly conserved in these species.[9-12] Previous studies of the human immune response to *Leptospira* indicate that LipL32 is the immunodominant protein antigen during infection,[12,13] and its use as a diagnostic target has looked promising in several studies with human and canine samples.[12,14-19] A recombinant LipL32-based antibody detection system is an attractive marker for use in serological tests for leptospirosis.

Because of the zoonotic potential of leptospirosis and the possibility of infected dogs serving as a reservoir for other dogs and humans, achieving a rapid diagnosis is of high importance. The potentially fatal consequences of inadequate therapy and the zoonotic risks related to handling dogs with leptospirosis create a need for a rapid, sensitive, and convenient diagnostic tool to be used at the point-of-care to detect antibodies against pathogenic *Leptospira* in dogs. The purpose of this study was to compare the LipL32-based SNAP Lepto to the MAT for detection of anti-*Leptospira* spp antibodies in the following groups: serum samples previously submitted for MAT testing from dogs with an unknown infection status and vaccination history, MAT negative sera from healthy dogs living in Alaska, canine sera positive for antibodies to *Borrelia burgdorferi*, and serial serum samples obtained from healthy dogs after *Leptospira* vaccination.

MATERIAL AND METHODS

Samples

Canine serum samples (n=460) that were submitted to IDEXX Reference Laboratories, Inc. (IRL) for MAT testing from locations throughout the United States were collected for this study. Of these, 201 samples were MAT negative and 259 samples were MAT positive. MAT positive samples had a titer of 1:100 or greater to one of the following 6 serogroups: Grippotyphosa, Canicola, Pomona, Icterohaemorrhagiae, Bratislava, and Autumnalis. Differentiation between the serovars in positive samples was accomplished by identification of the serovar associated with the highest agglutinating titer (peak MAT titer). All samples were divided into aliquots and stored at –20°C until use.

Serum samples (n-150) were obtained from a veterinary clinic located in Alaska, which is considered a *Leptospira* non-endemic region. These samples were drawn from healthy dogs with no known history of vaccination against leptospires. All samples were divided into aliquots and stored at –20°C until use.

Serum samples (n=52) from dogs with antibodies to *B burgdorferi* (Lyme Quant C6®)20 were obtained from a veterinary clinic located in Minnesota. The clinic does not vaccinate its patients against leptospirosis. However, travel and prior vaccination history was not available for these dogs. All samples were divided into aliquots and stored at –20°C until use.

Serum samples from healthy, client-owned dogs (n=28) with no history of *Leptospira* vaccination for at least 1 year were obtained from an IACUC approved study at Colorado State University.21 Inclusion criteria stipulated dogs be between 1 and 8 years of age, >15 kg, healthy, and have a known vaccination history with no *Leptospira* spp vaccination in the previous year. Dogs were randomly assigned to be administered one of four commercially available vaccinesb-e containing the Canicola, Grippotyphosa, Icterohaemorrhagiae, and Pomona serovars. All vaccinations were delivered subcutaneously at the left shoulder. After the initial vaccine administration (week 0), all dogs were administered the same vaccine within 3 days of week 3. Approximately 52 weeks later, dogs were administered the same vaccine as used at weeks 0 and 3. Blood was collected by jugular or cephalic venipuncture prior to vaccination on week 0 and then again approximately on weeks 3, 4, 7, 15, 29, 52, and 56. Sera samples from weeks 0, 3, 4, 52, and 56 were tested in this study. Sera were separated and stored at -20°C until tested.

Microscopic Agglutination Test

MAT was performed either at the IRL (percent agreement study, *Borrelia burgdorferi* antibody cross-reactivity study) or the Colorado State University Veterinary Diagnostic Laboratory (vaccine study population; www.dlab.colostate.edu) in accordance with their standard operating procedures. IRL testing utilized the following six serovars: Pomona, Canicola, Icterohaemorrhagiae, Grippotyphosa, Bratislava, and Autumnalis; Colorado State University testing utilized five of these serovars, substituting serovar Hardjo in place of Autumnalis. MAT was performed by mixing dilutions of canine sera (starting at 1:100) with live *Leptospira* organisms of each serovar. The highest dilution of serum in which >50% agglutination of organisms occured was reported as the titer for a given serovar. Samples that demonstrated agglutination to any of the serovars at any serum dilution were characterized as positive. Differentiation between the serovars in positive samples was accomplished by identification of the serovar associated with the highest agglutinating titer. MAT positive samples were characterized further by serovar and peak titer value. Samples that did not demonstrate agglutination with any of the serovars at a 1:100 serum dilution were characterized as negative.

SNAP Lepto

A rapid ELISA was used to detect antibodies against *Leptospira* in canine serum samples. This SNAP assay makes use of reversible chromatographic flow of sample and automatic, sequential flow of wash solution and

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Table 1. *SNAP Lepto performance with MAT positive samples by peak titer.*

Peak MAT Titer	# Samples	# Rapid ELISA Positive	% ELISA Positive
100	8	5	62.5%
200	20	11	55.0%
400	29	21	72.4%
800	53	37	69.8%
1600	34	25	73.5%
3200	13	10	76.9%
6400	19	16	84.2%
12800	32	29	90.6%
25600	14	14	100.0%
51200	18	18	100.0%
>102400	19	19	100.0%
Total	259	205	79.2%

enzyme substrate. Color development in spots on the flow matrix is used to provide the assay results, which are read visually.

The assay detects antibodies against *Leptospira* recombinant LipL32 protein.[11] Recombinant LipL32 was expressed and purified using conventional molecular techniques.[22] Polystyrene latex particles were covalently coupled to recombinant LipL32 and deposited on the flow matrix (sample spot). Additionally, recombinant LipL32 was conjugated with horseradish peroxidase (HRP). The assay also incorporates a positive control spot, which consists of antibodies to horseradish HRP and captures a small portion of the conjugate during the assay and turns blue upon addition of the substrate.

Briefly, 3 drops of test sample were mixed with 4 drops of the LipL32-HRP conjugate and deposited in the sample well. *Leptospira*-specific antibodies, if present in the sample, would bind to the LipL32–HRP in the conjugate. Immune complexes that formed would bind to the LipL32 coated particles on the solid phase (sample spot) of the ELISA. The ELISA was then exposed to wash solution and substrate reagents.

The presence or absence of antibody was determined by visual interpretation, comparing color intensities of the sample spot with the background color intensity of the flow matrix in the result window of the assay. Color in the sample spot that was greater than color of the background indicated that a sample was positive for antibody to *Leptospira*. A colorless sample spot indicated that a sample was negative for antibody to *Leptospira*. The positive control spot must have turned blue for the assay to be validated.

Statistical Methods

Statistical analysis was performed using standard formulas to calculate percent agreement between the MAT and SNAP Lepto (SAS® 9.3). All confidence intervals (Clopper-Pearson) are two-sided and calculated as 95% confidence intervals.

RESULTS

A population of 460 canine serum samples that had been submitted to the IRL by a veterinary clinic or hospital for MAT testing was obtained and tested with SNAP Lepto. Two hundred and fifty-nine samples were MAT positive with a titer >1:100 to one of six serogroups tested. Overall, SNAP Lepto yielded positive results for 205/259 samples (79.2% agreement; 95% confidence limits 73.7 - 83.9%). Percent agreement between SNAP Lepto and MAT increased with increasing peak MAT titer (Table 1). With

Table 2. SNAP Lepto performance with MAT positive samples by peak titer.

Serovar	# Samples	# Rapid ELISA Positive	% ELISA Positive
Autumnalis	17	13	76.5%
Bratislava	12	11	91.7%
Canicola	36	27	75.0%
Grippotyphosa	118	95	80.5%
Icterohaemorrhaigiae	16	12	75.0%
Pomona	60	47	78.3%
Total	259	205	79.2%

samples that had a peak titer suggestive of active infection, >1:3200,[4] the percent agreement between SNAP Lepto and MAT was 92.2% (106 of 115 samples; 95% confidence limits 85.7 – 96.4%). SNAP Lepto detected samples with peak MAT titers to each of the 6 serogroups tested, and performance was similar across each of these serogroups (Table 2).

Two hundred and one of the 460 canine serum samples obtained from IRL tested MAT negative and were also tested with SNAP Lepto. The percent agreement in testing this sample population was 82.1% (165 of 201 samples; 95% confidence limits 76.1 – 87.1%). In order to better understand the performance of the SNAP Lepto with MAT negative samples, 150 MAT negative samples from healthy dogs in a non-endemic area (Alaska) were also tested. The percent agreement in this population was 96.0% (144 of 150 samples; 95% confidence limits 91.5 – 98.5%).

Fifty-two canine serum samples that had positive results for *B burgdorferi* antibodies (Lyme Quant C6®<, IRL)[20] were tested on the IRL MAT and SNAP Lepto to assess cross-reactivity in dogs with another spirochaetal disease. This population spanned the range of Lyme Quant C6® values, from weak to strong. Ten of the 52 Lyme positive samples exceeded the reportable range of the Quant C6® assay. The range of Quant C6® values for the remaining 42 samples was 42-297 U/mL and the median value was 125 U/mL. The IRL MAT results for all 52

samples were negative for all six serogroups tested. Fifty-one of the 52 Lyme positive samples (98.1%) tested negative on SNAP Lepto. The one sample that tested positive on SNAP Lepto had a weak Quant C6® value of 44.

In order to evaluate whether SNAP Lepto detects antibodies resulting from *Leptospira* vaccination, samples from a study[21] at Colorado State University with healthy client-owned dogs were tested. Pre-vaccination samples (day 0) were tested by SNAP Lepto and MAT, and only samples with negative day 0 results on both tests were considered for further evaluation (n=28). Following initial vaccination on day 0, 27 of 28 dogs had a positive MAT titer at week 3, and 7 of these had peak titers of 1:3200.[21] Fifteen of the 28 dogs tested positive on SNAP Lepto, and all were also MAT positive. The number of SNAP Lepto positive dogs after the second vaccination at week 3 increased to 22 of the 28 dogs (tested at week 4). All 28 dogs were MAT positive at week 4, and the highest observed MAT titer increased to 1:6400 (n=7 dogs).[21] The number of antibody positive dogs decreased over time in this one year study. Samples from 21 of the 28 dogs were available one year post-vaccination. Three of 21 dogs had positive MAT titers, with 1:200 being the highest titer observed.[21] Five of 21 dogs tested positive on SNAP Lepto, and one of these was also MAT positive. A total of 19 dogs were administered one dose of the same vaccine approximately 1 year

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following the initial vaccination. Fifteen of these 19 dogs had positive MAT titers 4-6 weeks post-vaccination (weeks 56-60), with 1:1600 being the highest MAT titer observed (n=5 dogs).[21] Sixteen dogs were positive on SNAP Lepto, and 14 of these were also positive by MAT.

DISCUSSION

Definitive diagnosis of canine leptospirosis currently requires laboratory testing to detect the organism in clinical samples or use of the MAT.[4] Because of the zoonotic potential of leptospirosis and the potentially fatal consequences of inadequate therapy, there is great need for a rapid, sensitive, and convenient diagnostic tool to be used at the point-of-care. The current study compared the percent agreement of the LipL32-based SNAP Lepto to the MAT for detection of antibodies to canine pathogenic *Leptospira* spp. Agreement of SNAP Lepto in a population of MAT positive samples increased with increasing peak MAT titers, reaching 90.6% with peak titers >12,800. Agreement of SNAP Lepto with MAT negative samples was 82.1% in samples submitted to IRL for MAT testing and was highest with samples from healthy dogs (96.0%). Both SNAP Lepto and the MAT detected antibodies induced by *Leptospira* vaccination.

The canine serum samples submitted to the IRL for MAT testing were presumably obtained from sick dogs suspected of having leptospirosis. However, because of the lack of patient histories, the lack of access to convalescent MAT results, and the lack of vaccination history, the true clinical status of the patients cannot be determined. Although a single positive titer can serve to increase suspicion for leptospirosis, even a high titer does not confirm a diagnosis.[3] In dogs with a known history of vaccination, this is particularly important because although postvaccinal titers tend to be low, titers > 1:1600 have the potential to persist after vaccination.[23] For these reasons, the current study only compared performance of the point-of-care ELISA to MAT status and peak MAT titer without any attempt to interpret

clinical status of each dog.

In testing the MAT positive canine serum samples from the IRL, 21.8% tested negative on SNAP Lepto. These discrepant results may represent false negative SNAP Lepto results, whereby these discrepant samples may contain agglutinating antibodies in the absence of detectable anti-LipL32 antibodies on SNAP Lepto. In the early stages of infection, dogs may frequently have negative serology results.[3,4] It is for this reason that the current recommendation for MAT testing is to submit acute and convalescent samples, looking for a 4-fold change in titers in order to make a definitive diagnosis.[3] It is unknown whether repeated testing of samples from dogs with these discrepant results would later yield positive SNAP Lepto results. In suspected cases of canine leptospirosis where the SNAP Lepto result is negative, yet the level of suspicion remains high due to clinic signs, use of the MAT and/or polymerase chain reaction (PCR) assays are valuable adjuncts to the diagnosis of leptospirosis. Further clinical studies that include cases defined by acute and convalescent MAT titers, as well as PCR, are required to understand this discrepancy.

In testing of MAT negative canine serum samples from the IRL, 17.9% were positive on SNAP Lepto. These samples were originally submitted for MAT testing, and presumably obtained from sick dogs suspected of having leptospirosis. These discordant results may represent false negative MAT results. In contrast, samples from healthy, non-vaccinated dogs in Alaska produced a significantly lower positive rate (4.0%) on SNAP Lepto. These data suggest that MAT negative, SNAP Lepto positive results are more common in dogs suspected of having leptospirosis, and that these discordant results may be the result of false-negative MAT results. MAT titers typically become positive after approximately 1 week and peak after 3-4 weeks.[4] Because initial positive MAT results can take a week or more to develop, negative initial MAT results are not

uncommon. It is for this reason that acute and convalescent testing is the recommended protocol for MAT testing.[3] Unfortunately, paired acute and convalescent samples were not available from these dogs to allow for subsequent testing. Another possible explanation for the discordant results is that these samples may have been obtained from dogs later in the disease, when the level of circulating agglutinating antibodies tends to decrease.[23]

An alternative explanation for these MAT negative, SNAP Lepto positive discordant results is that these samples contain anti-LipL32 antibodies but lack agglutinating antibodies detectable by MAT. These results could occur as a result of exposure to *Leptospira* organisms in the environment or prior *Leptospira* vaccination. The present study demonstrates that some currently vaccinated dogs tested positive on SNAP Lepto for a varying amount of time post-vaccination. Complete vaccine histories were not available for patients included in this study, and it is possible that *Leptospira* vaccination produced positive results on SNAP Lepto in this study. Detection of antibodies induced by vaccination is a limitation to interpretation of all *Leptospira* serological tests, including the MAT, particularly when only an acute titer is obtained. Paired MAT titers may facilitate the interpretation of the results if vaccinal antibodies are present. [21,23,24]

Finally, these MAT negative, SNAP Lepto positive discordant results could represent false-positive SNAP Lepto results. False-positive SNAP Lepto results could occur from cross-reactivity with homologous antigens or infection with other pathogens. LipL32 is expressed only by pathogenic *Leptospira*,[9,11,12,25] and the only ortholog identified to date is in the unrelated marine bacterium *Pseudoalteromonas* tunicata.[26] However, the existence of undiscovered homologous antigens cannot be ruled out. Furthermore, data obtained in testing the MAT negative, Lyme positive samples supports the lack of cross-reactivity with antibodies against another common pathogenic

spirochete, *B burgdorferi*.

Leptospirosis can be a challenging disease to diagnose. The microscopic agglutination test (MAT) for detection of antibodies is the most common diagnostic test used today to aid in the diagnosis of canine leptospirosis.[3,4,6] The MAT has some limitations due to the inability for it to be run as point-of-care assay. The primary advantage of a rapid ELISA, such as SNAP Lepto, is the convenience of performing the test in a hospital or reference laboratory setting without the need for expensive cultures and equipment. This makes a serologic test for leptospirosis more available to practitioners in a hospital setting, thereby ensuring adequate precautions are taken to reduce the risk of handling dogs with a zoonotic disease, as well as address the need to administer adequate therapy in a timely manner. SNAP Lepto also provides the ability to run consecutive tests over a matter of days in order to detect seroconversion in a patient, often prior to receiving results of an initial sample submission for MAT testing. However, future study with clinically defined canine leptospirosis cases is required to evaluate the potential utility of SNAP Lepto in repeated convalescent sample testing.

In summary, the current study describes the performance of the LipL32-based SNAP Lepto for detection of anti-LipL32 antibodies. This test does not distinguish between serovars, including vaccinal versus non-vaccinal serovars, and does not provide a titer magnitude, and these factors must be taken into consideration when interpreting the test results. However, SNAP Lepto provides a convenient tool to assess *Leptospira* antibody status in dogs, thereby providing potentially valuable information to this complex diagnostic work up.

FOOTNOTES

a Lyme Quant C6®, IDEXX Laboratories, Inc., Westbrook, ME.

b LeptoVax 4, Boehringer-Ingelheim, St. Joseph, MO

c Nobivac Lepto4, Merck Animal Health,

Whitehouse Station, NJ

d RECOMBITEK 4 Lepto, Merial, Duluth, GA

e Vanguard L4, Pfizer Animal Health, New York, NY

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