SHORT COMMUNICATIONS

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Evaluation of Two Serologic Tests for Rapid Detection of Anti-*Leptospira* Antibodies in California Sea Lions (*Zalophus californianus*)

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ABSTRACT: Leptospirosis is a bacterial zoonosis impacting wild and domestic animals globally. *Leptospira interrogans* serovar Pomona is endemic in free-ranging California sea lions (CSLs; *Zalophus californianus*), and leptospirosis is frequently diagnosed in stranded CSLs. Serum microscopic agglutination test (MAT) is a commonly performed diagnostic assay, and CSLs with clinical disease have reliably elevated MAT titers. However, MAT results may not be available for several days after sampling. Given the zoonotic and high transmission potential of *Leptospira* spp., a point-of-care diagnostic test would be valuable in rehabilitation and managed care settings and during outbreak response efforts. The ID SNAP and Zoetis WITNESS anti-*Leptospira* antibody tests are rapid diagnostic tools that have been validated in dogs and give a qualitative (positive or negative), not quantitative (exact titer), result. The SNAP test uses ELISA to detect both immunoglobulin (Ig)M and IgG antibodies, whereas the WITNESS test is a lateral flow assay that only detects IgM. We compared SNAP and WITNESS results with MAT results by using serum collected from stranded and free-ranging CSL with negative, low, medium, and high anti-*Leptospira* antibody titers as previously determined by MAT. Percent agreement between SNAP and MAT results was high, with a Cohen's kappa statistic of 0.957. No WITNESS tests were positive. These findings suggest that the SNAP test may be useful for detecting anti-*Leptospira* antibodies and ruling out leptospirosis in CSL.

Key words: California sea lion, Lepto SNAP, Lepto WITNESS, *Leptospira*, leptospirosis, microscopic agglutination test, *Zalophus californianus*.

Leptospirosis is an infection caused by bacteria of the genus *Leptospira* that impacts wild and domestic animals globally. *Leptospira interrogans* serovar Pomona is endemic in free-ranging California sea lions (CSLs; *Zalophus californianus*) and causes yearly, seasonal epizootics with large epizootics every 3–5 yr (Gulland et al. 1996; Lloyd-Smith et al. 2007; Prager et al. 2020). Historically during epizootics, as many as 350 CSLs have stranded with leptospirosis along the 965-km (600-mi) range of The Marine Mammal Center (TMMC; Sausalito, California, USA). Typically, CSL with leptospirosis present with clinical signs of hyporexia, lethargy, polydipsia, and perceived abdominal discomfort (Gulland et al. 1996; Prager et al. 2013) as well as serum biochemical changes associated with renal compromise (e.g., azotemia, hypernatremia, hyperphosphatemia, and hypokalemia; Gulland et al. 1996; Whitmer et al. 2021).

Definitive diagnosis of leptospirosis can be obtained using PCR to detect pathogenic *Leptospira* DNA, culture, detection of leptospiral antigens in clinical material, and immunohistochemistry. These tests are relatively costly and time consuming and require samples that may be difficult to obtain antemortem, so they are not routinely performed during marine mammal rehabilitation. For CSLs in rehabilitation, presumptive diagnosis is often made based on pathognomonic clinical signs and serum biochemical changes. Although differential diagnoses—including urogenital carcinoma, nephrolithiasis, and amyloidosis—should be considered, leptospirosis is the most common nephropathy observed in stranded CSLs (Greig et al. 2005). Renal ultrasonography can be helpful, particularly in screening for hydroureter and hydronephrosis associated with urogenital carcinoma (Whitmer et al. 2021).

Serum antibody titers, evaluated by microscopic agglutination test (MAT), are another useful tool for diagnosing leptospirosis. Although other serologic tests for anti-*Leptospira* antibodies are available, the MAT is the test used by

marine mammal rehabilitation centers to aid in the diagnosis of leptospirosis in CSLs (Colagross-Schouten et al. 2002). Although a positive MAT only definitively indicates prior exposure, diagnosis can be inferred when coupled with clinical signs, antibody titer magnitude, and serum biochemical changes suggestive of leptospirosis (Prager et al. 2020; Whitmer et al. 2021), and if other differentials have been ruled out. Quantitative MAT anti-*Leptospira* antibody titers can be helpful in determining infection status. The probability that an animal is PCR-positive increases with antibody titer: CSLs with titers of 1:51,200 or greater have a shedding probability >0.8 (Prager et al. 2020). The majority of stranded CSLs that have clinical signs associated with leptospirosis have antibody titers in this range; this is probably because the time between exposure to *Leptospira* spp. and the development of severe leptospirosis is sufficient to generate a robust immune response (Prager et al. 2020). Low positive titers in stranded CSLs without renal disease probably represent historic infections from which they have recovered, that is, they are no longer infected (Prager et al. 2020).

Two rapid tests are commercially available and widely used for point-of-care diagnosis of leptospirosis in canines. The IDEXX SNAP test (IDEXX, Westbrook, Maine, USA) detects *Leptospira*-specific immunoglobulin (Ig)G and IgM antibodies and is an ELISA based on LipL32, the dominant protein antigen of pathogenic *Leptospira* (Curtis et al. 2015). Evaluation of the SNAP test by using canine sera has shown that the likelihood of agreement of the SNAP test with MAT results increases with higher MAT titers and has shown 100% agreement with MAT titers >12,800 (Curtis et al. 2015). The Zoetis WITNESS test (Zoetis, Parsippany, New Jersey, USA) is a lateral flow assay for detection of IgM antibodies to several leptospiral serovars, including *L. interrogans* serovar Pomona—the serovar seen in CSLs. In canines, it has a reported sensitivity of 98% and specificity of 93.5% relative to detection of antibodies via MAT (Kodjo et al. 2016). Anti-*Leptospira* IgM is detectable within the first 3 d of infection in humans (Sykes et al. 2011), and previous research in dogs has demonstrated that the WITNESS test has a higher sensitivity early in infection than MAT and the SNAP test (Lizer et al. 2018).

Our study aimed to determine whether the SNAP and/or WITNESS tests would be useful for diagnosis of leptospirosis in CSLs. Because CSLs that become ill enough with leptospirosis to strand and enter rehabilitation have probably been infected longer than domestic animals in which illness is often detected by the owner soon after infection, we predicted that IgG levels in stranded CSLs would be detectable, whereas IgM levels might have already declined below detection. We hypothesized that there would be strong agreement between the results of the SNAP test and MAT, especially for samples from individuals with higher MAT titers, but no-to-slight agreement between the results of the WITNESS test and MAT.

We selected banked sera from stranded CSLs (n=45) with previous positive MAT results ranging from low (1:100) to high (1:819,200) titers. These samples had been collected for routine diagnostics from CSLs during rehabilitation at TMMC between 2017 and 2022. Animals with negative MAT results (negative at a dilution of 1:100, n=15) were selected from a group of wild-caught CSLs sampled on San Miguel Island, San Nicolas Island, or Año Nuevo Island, California, USA, during a period of known *L. interrogans* serovar Pomona infection fadeout from the population (i.e., between 2013 and June 2017; Lloyd-Smith and Prager 2021). All wild CSL samples had been collected under National Marine Fisheries Service permits 17115, 21422, and 24359. The sample collection protocol was approved by the Institutional Animal Care and Use committees of TMMC (protocol 2021-1-1) and the University of California–Los Angeles (ARC 2012-035-12 and 2012-035-23).

For sample acquisition, whole blood was collected from anesthetized or physically restrained individuals directly into Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and centrifuged to separate serum. This serum was stored at −20 C or −80 C. Serum samples from the stranded CSLs (n=45) were categorized into low, medium, and

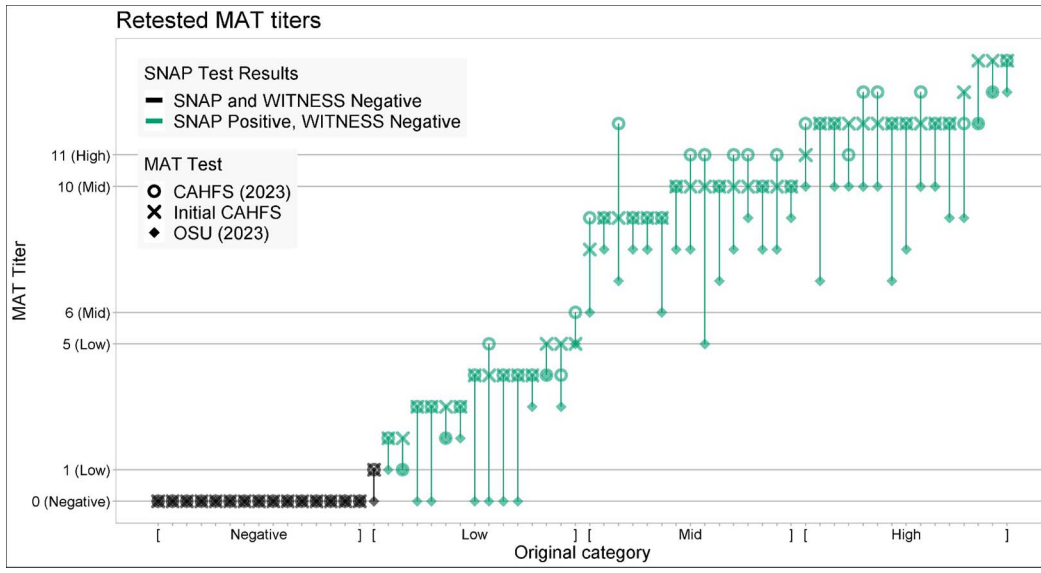

FIGURE 1. Initial and repeat microscopic agglutination test (MAT) results to detect anti-*Leptospira* antibodies in California sea lion (CSLs; *Zalophus californianus*) serum samples assigned to low, medium, and high MAT titer categories. Initial MAT had been performed at California Animal Health and Food Safety Laboratory, Davis, California, USA (CAHFS), after serum acquisition from wild-caught and stranded CSLs between 2013 and 2022 and was used for sample categorization. Repeat MAT was performed at both CAHFS and at College of Veterinary Medicine, Oregon State University (OSU) Corvallis, Oregon, USA, in 2023. The MAT titers have been log transformed ($1+\log_2(\text{titer}/100)+1$), thus 1:100=1, 1:200=2, etc.).

high titer categories. Categorization was based on previously obtained MAT results (California Animal Health and Food Safety Laboratory, Davis, California, USA [hereafter CAHFS]), with low titers categorized as 1:100–1:1,600; medium titers as 1:3,200–1:51,200; and high titers as ≥1:102,400. These groups were defined based on previous categorization and the association of titers with PCR confirmed infection (Prager et al. 2020).

Frozen serum (minimum volume, 1.5 mL) samples from each individual CSL (n=60) were thawed and split into three 0.5-mL aliquots. Aliquoted samples were refrozen such that each underwent the same freeze-thaw cycle before testing. One aliquot from each individual was used for the MAT at CAHFS and one for the MAT at the Oregon State University (OSU) College of Veterinary Medicine (Corvallis, Oregon, USA); the third was used to perform the SNAP and WITNESS rapid tests at the diagnostic laboratory at TMMC.

Trained laboratory personnel performed the SNAP and WITNESS tests on the same day and according to manufacturer's instructions. A single individual performed each test type (i.e., one person performed every SNAP test and another person performed every WITNESS test). Positive, negative, and overall percent agreement were calculated between the initial CAHFS MAT results (i.e., qualitative positive or negative MAT results) and each rapid test. Percent agreement was also calculated between repeat MAT results performed in 2023 at CAHFS and OSU, and initial CAHFS MAT results. We evaluated the level of agreement between the MAT results and the rapid tests by estimating Cohen's kappa statistic (Cohen 1960). Data analysis was performed using Prism 10 software (GraphPad Software, Boston, Massachusetts, USA) and R 4.2.2 statistical software (R Core Team 2023). Cohen's kappa calculation was performed via the psych package in R (Revelle 2017). We plotted MAT results by using R statistical software (Fig. 1).

Overall percent agreement of the SNAP test with initial MAT (CAHFS) results was

98.3% (positive percent agreement, 97.8%; negative percent agreement, 100.0%), with a calculated Cohen's kappa statistic of 0.957 (Table 1). When percent agreement was evaluated for each MAT titer category, the SNAP tests were negative for all samples in the negative category, positive for 14/15 (93%) samples in the low titer category, and positive for all the samples in the medium and high MAT titer categories (Table 2).

The WITNESS tests were negative for all 60 individuals. The overall percent agreement with the MAT results was 25.0% (positive percent agreement, 0.0%; negative percent agreement, 100.0%; Table 1), with a Cohen's kappa statistic of 0.0, indicating no agreement between the two tests (Table 1). When percent agreement was evaluated for each MAT titer category, the WITNESS tests in the negative category had 100% agreement with MAT and the low, medium, and high categories had no agreement (Table 2). Although the SNAP test is capable of detecting both IgG and IgM antibodies, the WITNESS test detects only IgM (Lizer et al. 2018). Part of the primary immune response, IgM is relatively short lived, peaking at ~2 wk after infection in dogs (Lizer et al. 2018). Hence, we hypothesize that the lack of agreement between the WITNESS and MAT results is due to the length of time between initial infection and testing in stranded CSLs. Although the precise longevity of IgM in CSLs serum following acute leptospirosis infection is unknown, the WITNESS results suggest that the individuals included in this study were sampled after IgM production had declined.

Percent agreement was calculated between initial CAHFS MAT results and MAT results performed at both CAHFS and OSU after the same freeze-thaw cycle in 2023. Because of known issues of interreader, interlab, and intertest run variation of up to a few dilutions (Mummah et al. 2024), the two tests were considered to agree when they varied by no more than two dilutions. Agreement between initial CAHFS and 2023 CAHFS results was 100% for negative, low, and high categories and 93% (14/15) for the medium category (Table 2). Agreement between the initial CAHFS and 2023 OSU results was 100% for negative, 60% (9/15) for low, 80% (12/15) for medium, and 67% (10/15)

TABLE 1. Results of IDEXX SNAP tests and Zoetis WITNESS tests to detect anti-*Leptospira* antibodies in CSL (*Zalophus californianus*) serum and measures of agreement between these two rapid tests and MAT results. The MAT had been performed at California Animal Health and Food Safety Laboratory after serum acquisition from wild-caught and stranded CSLs between 2013 and 2022.[a]

Rapid test	Qualitative result	MAT			Overall % agreement (95% CI)	Positive % agreement (95% CI)	Negative % agreement (95% CI)	Cohen's kappa statistic (95% CI)
		Positive	Negative	Total				
IDEXX SNAP test	Positive	44	0	44	98.3 (90.3–100.0)	97.8 (87.4–100.0)	100.0 (76.1–100.0)	0.957 (0.872–1.000)
	Negative	1	15	16				
	Total	45	15	60				
Zoetis WITNESS test	Positive	0	0	0	25.0 (15.7–37.3)	0.0 (0.0–9.4)	100.0 (76.1–100)	0.00 (0.00–0.00)
	Negative	45	15	0				
	Total	45	15	60				

[a] CSL = California sea lion; MAT = microscopic agglutination test; CI = confidence interval.

TABLE 2. Percent agreement between results of serologic tests to detect anti-*Leptospira* antibodies in California sea lion (*Zalophus californianus*) serum: initial MAT results performed at CAHFS after sample acquisition from wild-caught and stranded CSLs between 2013 and 2022 are compared with results of IDEXX SNAP, Zoetis WITNESS, MAT performed at CAHFS in 2023, and MAT performed at College of Veterinary Medicine, OSU, in 2023. Results were separated into negative, low, medium, and high categories based on initial CAHFS MAT results.[a]

Serologic category	% agreement with initial MAT (CAHFS)			
	IDEXX SNAP (95% CI)	Zoetis WITNESS (95% CI)	MAT (CAHFS 2023) (95% CI)	MAT (OSU 2023) (95% CI)
Negative	100 (76.1–100)	100 (76.1–100)	100 (76.1–100)	100 (76.1–100)
Low	93 (68.2–100)	0 (0–23.9)	100 (76.1–100)	60 (35.6–80.3)
Medium	100 (76.1–100)	0 (0–23.9)	93 (68.2–100)	80 (54.1–93.7)
High	100 (76.1–100)	0 (0–23.9)	100 (76.1–100)	67 (41.5–85)

[a] MAT = microscopic agglutination test; CAHFS = California Animal Health and Food Safety Laboratory, Davis, California, USA; OSU = Oregon State University; CI = confidence interval.

for high categories (Table 2). Agreement between 2023 CAHFS and 2023 OSU results was 100% for negative, 60% (9/15) for low, and 47% (7/15) for both medium and high categories. The differences in 2023 MAT titers included values higher and lower than the initial MAT values and are therefore not consistent with sample degradation, which would result in titers being consistently lower than upon initial sampling (Fig. 1). Such intertest and interlaboratory MAT titer variation has been previously reported previously, and it is probably due to differences in lab conditions, materials, and personnel and is consistent with prior reports that variation of 1–2 dilutions may be expected when repeat testing occurs (Chappel et al. 2004; Sykes et al. 2011; Mummah et al. 2024).

Our results indicate that the IDEXX SNAP test agrees strongly with MAT results for detection of anti-*Leptospira* antibodies in CSL serum. This suggests that the SNAP test may be a valuable rapid diagnostic tool to rule out *Leptospira* infection in sea lions in a rehabilitation setting or for rapid surveillance during an outbreak response. It may also be a valuable diagnostic for infection in CSLs in managed care if prior serum samples are available and seroconversion is detected. This test must be interpreted with caution, however, because a positive result cannot differentiate between active infection, past (cleared) infection, or chronic subclinical infection (Prager et al. 2020). All the individuals used in this study that fell within the low titer category, for example, presented for rehabilitation with other disease processes, and their low positive titers were probably due to either historic infection or chronic subclinical infection. In these patients, their exposure to *Leptospira* was unlikely to be of clinical significance at the time of stranding. In a managed care setting, comparing SNAP test results in a clinically ill patient with previously negative SNAP and/or MAT results to detect seroconversion over a short time period would allow for diagnosis of acute *Leptospira* infection. Further research is needed to evaluate whether the WITNESS test may be useful to detect acute infection in marine mammals in settings where illness may be observed sooner, such as in a managed care setting.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-24-00055.

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