TECHNICAL BULLETIN

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WITNESS® Lepto Test Kit for Detection of *Leptospira* IgM in Dogs

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Canine leptospirosis, an acute bacterial infection caused by pathogenic spirochetes of the genus *Leptospira*, is an infectious disease of dogs with worldwide distribution. Because infected dogs excrete leptospires in their urine, they pose a potential zoonotic risk to humans. Leptospirosis should be part of the differential diagnosis whenever dogs present with acute fever of unknown origin, unexplained renal or hepatic disease, thrombocytopenia, anterior uveitis, pulmonary hemorrhage, or abortion. In-clinic identification of infected dogs may be achieved early in the course of a *Leptospira* infection by detection of immunoglobulin M (IgM) antibodies against *Leptospira* in anti-coagulated whole blood, serum, and plasma by immunoassay. The WITNESS® Lepto Test Kit from Zoetis Diagnostics is an accurate and reliable Rapid Immuno Migration (RIM™) test designed to provide veterinarians with a point-of-care solution for quickly and confidently identifying dogs with IgM antibodies associated with the presence of *Leptospira* spp. The test is sensitive and specific, capable of detecting IgM antibodies to *Leptospira* serovars Grippotyphosa, Icterohaemorrhagiae, Canicola, and Pomona. As a screening diagnostic, WITNESS® Lepto requires only 5 µL of sample for each test and approximately 10 minutes to produce results. Data have demonstrated that the WITNESS® Lepto test performs favorably with the reference standard microscopic agglutination test (MAT). This technical bulletin reviews key details about leptospirosis, examines RIM™ technology, highlights how the WITNESS® Lepto Test Kit works, and provides supporting study data demonstrating test sensitivity and specificity.

SECTION 1.

OVERVIEW OF CANINE LEPTOSPIROSIS

Leptospira Infection

Canine leptospirosis, a disease of worldwide distribution and significant zoonotic potential, is an acute bacterial infection of dogs caused by pathogenic spirochetes belonging to the genus *Leptospira*.[1,2] *Leptospira* spp. are slender, tightly coiled Gram-negative bacteria (**Figure 1**) composed of an outer envelope including outer membrane proteins, a cell wall, and two flagella.[3] The flagella facilitate penetration of intact mucous membranes by causing the bacteria to roll in a manner similar to a corkscrew. The bacteria have pointed ends, which are usually bent into a distinctive hook.[1,4] In nature, leptospires survive for extended periods in wet and warm environmental conditions but are readily killed by drying and at temperature extremes.[3] At least 12 pathogenic and 4 nonpathogenic species, with more than 250 pathogenic serovars of *Leptospira* are currently recognized.[5] Leptospires were classified initially into serogroups on the basis of surface antigens, with each serogroup containing one or more serovars.[1,2,6] Newer molecular classification schemes divide the *Leptospira* genus into several species on the basis of DNA relatedness,[6–10] but currently epidemiologists and veterinarians continue using serogroup and serovar designations with which they are familiar.[1,2] Each *Leptospira* serovar has a definitive host that maintains the organism and is vital to its dissemination in the environment.[2] Definitive or maintenance host animals typically become infected at a young age,[11] and harbor and shed leptospires without manifesting clinical disease.[2,12] Infection in dogs is often associated with the presence of wildlife maintenance hosts (e.g., mice, rats, voles, possums, skunks, raccoons, deer) in the confines of urban areas.[13–15]

Prior to 1960, two *Leptospira* serovars—Canicola and Icterohaemorrhagiae—were regarded as the major causes of canine leptospirosis.[16] Dogs and rats are the maintenance hosts of serovars Canicola and Icterohaemorrhagiae, respectively.[17] Following widespread use of bivalent vaccines containing these serovars in the 1970s, the incidence of canine leptospirosis decreased markedly in Europe and North America.[11,17–26] Beginning in the 1990s, however, *Leptospira* serovars Pomona, Grippotyphosa, and Bratislava were increasingly identified in infected dogs, with the relative proportion of the serovars differing geographically.[18,20,26–29]

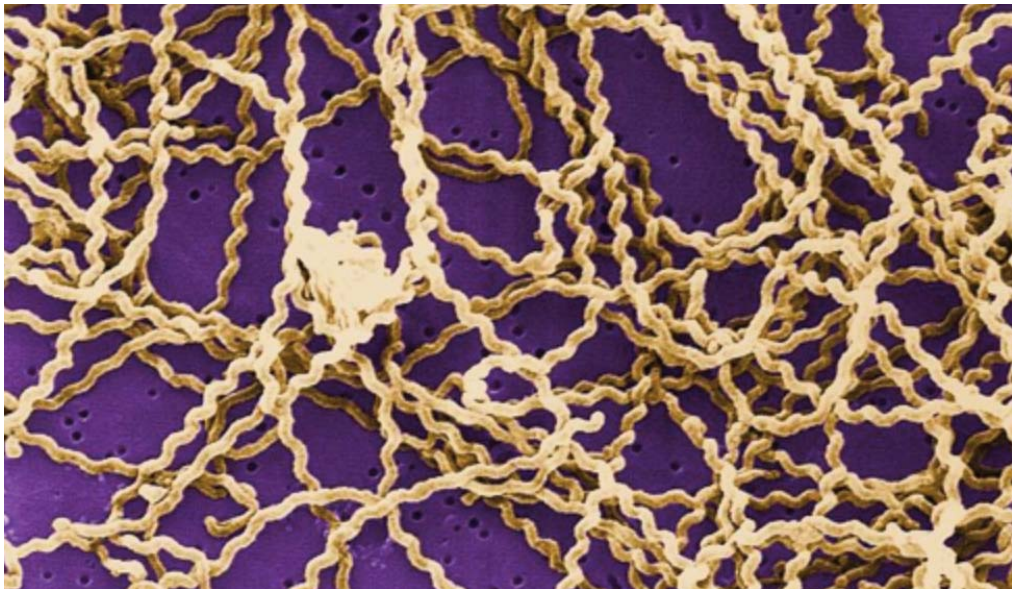

Figure 1. Scanning electron micrograph of *Leptospira* sp. bacteria. Leptospires generally share the same physical characteristics: tight coil, slender appearance, and curved hooks at one or both ends. (Source: Content Providers: CDC / Rob Weyant; Photo Credit: Janice Haney Carr.)

Although vaccination was associated with a substantial reduction in the incidence of disease in dogs caused by serovars Canicola and Icterohaemorrhagiae, dogs remained susceptible to disease caused by other serovars they encountered as urban expansion extended into areas previously considered rural.[30] In these habitats, dogs have greater opportunity for contact with wildlife, livestock, and livestock waste, which can serve as reservoirs for the three recently identified serovars.[11,20,21,23,26,31] The change in leptospirosis epidemiology is noteworthy because infection with wildlife and livestock *Leptospira* serovars can be associated with clinical disease of greater severity.[18] Whereas canine leptospirosis was once considered a rural disease that affected mainly large breed dogs, now, in urban environments, dogs of all breeds and sizes can be at risk.[11,30–32]

Optimal conditions for the survival of leptospires in nature include wetness, moderate temperatures (0°–25°C; 32°–77°F), and mildly alkaline soil. A higher seasonal incidence of disease has been observed in the summer and early fall, with more cases occurring in the southern semitropical belt of the U.S. and similar climatic regions worldwide.[33] Seasonality in many parts of North America is associated with rainfall, with reports of disease outbreaks occurring during or immediately after periods of flooding.[12,25,26,34] In warmer climates, where rapid desiccation would inhibit survival of leptospires in the environment, the incidence of disease is higher during rainy seasons.[15] Leptospires are transmitted between hosts by direct contact, venereal and placental transfer, bite wounds, or consumption of infected meat. Indirect transmission occurs through exposure of susceptible animals to contaminated water, soil, vegetation, food, and bedding.[33]

Pathogenesis

Leptospires enter the body via penetration of intact mucous membranes or abraded skin and begin rapidly replicating in the bloodstream for the next 4 to 12 days, depending on the virulence of the organism and the immune response of the host. Nonspecific clinical signs associated with this leptospiremic stage of infection can include fever, depression, anorexia, and generalized pain. Also developing during this time may be vasculitis, thrombocytopenia, and coagulopathy.[35] Through the bloodstream, leptospires invade the kidneys, liver, lungs, spleen, central nervous system, eyes, and reproductive tract. Damage to internal organs is dependent upon virulence of the infecting serovar and host susceptibility (**Figure 2**).[2,17,33,35,36] In a naïve, susceptible animal, leptospires that evade phagocytosis replicate exponentially, with a doubling time of approximately 8 hours within the bloodstream and tissues.[36] Replication ceases with either the appearance of specific antibody in the plasma and subsequent specific immune clearance, or death of the host.[36] IgM antibodies usually appear within 3 to 10 days of infection and react broadly with a variety of serovars, followed by the appearance of IgG antibodies.[37]

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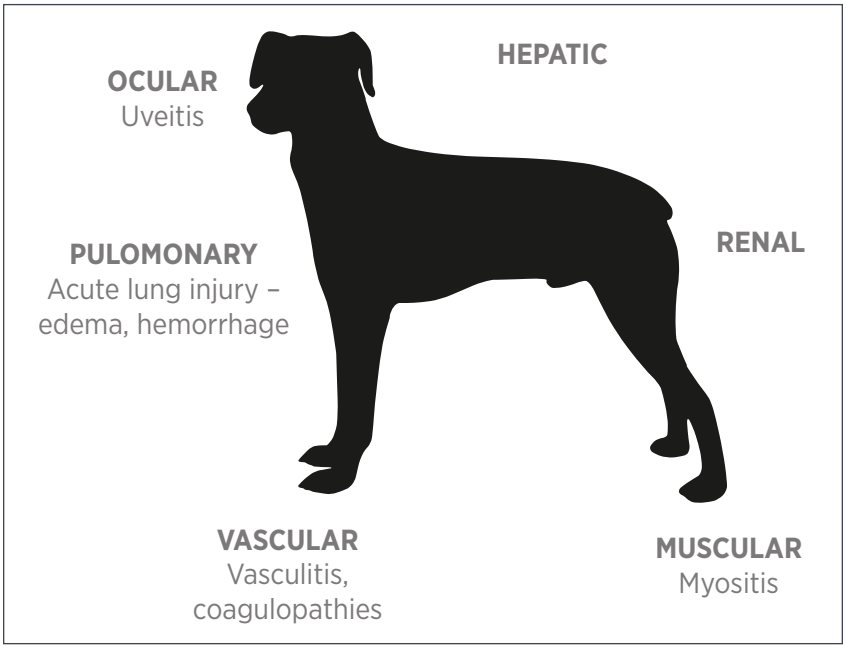


Figure 2. Clinical manifestations of canine leptospirosis.

Clinical Signs

Canine leptospirosis may present as peracute, acute, subacute, or chronic disease (**Table 1**). Clinical signs depend on age and immune status of the host, environmental factors, and virulence of the infecting serovar. Peracute infections are characterized by leptospiremia, shock, and often death with minimal clinical signs. Acute leptospirosis initially may be manifest by fever and muscle tenderness accompanied by tachypnea, tachycardia, decreased capillary perfusion, vomiting, dehydration, and shock. Terminally ill dogs become depressed and hypothermic, without time to develop renal and hepatic failure. More recently, European investigators have associated pathogenic leptospires with an acute pulmonary hemorrhagic syndrome in dogs,[38,39] but have not yet determined whether pulmonary abnormalities are attributable to autoimmune mechanisms or to exposure of circulating toxins produced by the pathogen at distant sites, such as the liver.[1,39] Subacute infection is the most common form of canine leptospirosis, but because veterinarians in many developed countries perceive leptospirosis to be rare, and because infection can be manifest with a myriad of symptoms, it is often underdiagnosed.[40–42] Initial clinical signs include vomiting, fever, and dehydration, followed by shock. Signs of coagulopathies—petechial and ecchymotic hemorrhages—may occur. Conjunctivitis, tonsillitis, and rhinitis may be accompanied by coughing and dyspnea. Signs of renal disease including polyuria, polydipsia, oliguria, or anuria may occur. Hepatic inflammation induces cholestasis, which may lead to acholic feces.[11] Chronic leptospirosis is an exacerbation or prolongation of the clinical signs found in the subacute form of the disease. Chronically infected dogs may develop a carrier condition in which leptospires replicate and remain sequestered in immunologically privileged sites such as the renal tubules and reproductive tract where they are protected against circulating antibodies. These animals shed large numbers of leptospires in their urine for months to years after infection and represent the primary route of leptospirosis distribution to other animals and to humans.[40]

Diagnosis

Timely diagnosis of canine leptospirosis is important because antibiotic therapy provides the greatest benefit to the patient when initiated early in the course of disease and to humans and other animals by reducing the zoonotic potential of the disease. Identification of early-phase leptospirosis, however, is

Table 1.
Clinical syndromes and signs of canine leptospirosis

Syndrome	Clinical Signs
Peracute	Massive leptospiremia causing shock, often fatal, with few premonitory signs
Acute	INITIAL: pyrexia, shivering, generalized muscle tenderness SUBSEQUENT: vomiting, rapid dehydration, shock, icterus
Subacute	Fever, anorexia, vomiting, dehydration, thirst, conjunctivitis, rhinitis, tonsillitis, coughing, dyspnea, reluctance to move
Chronic	RENAL FUNCTION: progressive deterioration accompanied by weight loss, polyuria, polydipsia, anorexia, vomiting HEPATIC FUNCTION: possible liver failure accompanied by inappetence, weight loss, ascites, icterus, hepatoencephalopathy

hampered by its non-specific clinical presentation.[40] Laboratory diagnosis of leptospirosis can be made either by detection of the leptospires or their nucleic acid (DNA) or antigens in blood, CSF, urine, and tissues, or by serological tests that detect leptospiral antibodies. The techniques currently available for direct detection of organisms or their DNA include dark-field microscopy, culture, and PCR assay.

- **Dark-field microscopy.** Visualization of leptospires by dark-field examination of body fluids (blood, urine, CSF, peritoneal fluid) is widely regarded as a method that lacks both sensitivity and specificity.[1,2,14] Approximately 10^4 leptospires/mL are required for one cell per field to be visible by dark-field microscopy.[14,15] Reportedly, even experts can occasionally confuse threads of fibrin and protein in wet preparations with leptospires.[14,37]
- **Culture.** Bacterial culture of blood or urine is the most definitive method for identifying infective *Leptospira* spp., but culture is difficult to perform and is constrained by slow growth rates of some *Leptospira* strains, necessitating long incubation periods before an isolate is established in culture.[5] Successful isolation requires fresh blood, urine, or tissue samples, which must be obtained before initiating antibiotic treatment, usually inoculation of at least two 10-fold dilutions of tissue fluid or homogenate and selective antimicrobial agents to inhibit contaminants.[14,37] Cultures must be incubated for up to 13 weeks at 30°C (86°F) with weekly examination by dark-field microscopy before they can be discarded as negative.[5] For these reasons, culture is not routinely used for diagnosing leptospirosis in individual dogs; however, the technique remains important for epidemiological reasons because it enables accurate identification of infecting serovars.[5]
- **PCR assay.** Testing using PCR analysis is becoming a more common modality for early identification of leptospirosis. Real-time PCR is the most sensitive PCR technique commercially available in some laboratories. Whole blood and urine samples can be tested simultaneously because blood samples tend to be positive early in the course of infection and then later the urine samples become positive.[2] Investigators in Europe have shown that PCR testing performed on canine blood, urine, or kidney samples correlated with results obtained with classic *Leptospira* culturing and microscopic agglutination testing (MAT).[43] Their work also showed that as early as 4 days after infection, blood culturing and PCR were positive, whereas the MAT was negative, presumably because it was too early for formation of serogroup-specific microscopic agglutinating antibodies.[43] At this early stage of disease, more cross-reactive genus-specific IgM antibody predominates.[15] A recent study has shown that PCR may also be used in dogs recently vaccinated against leptospirosis, as two real-time assays were not influenced by vaccinal DNA in the diagnosis of leptospirosis.[44] Not all PCR assays are alike, however, and they vary considerably in performance. Currently, limited information is available on the validity, sensitivity, specificity, and positive predictive value of the various PCR assays.[45]

Investigators in Europe have shown that as early as 4 days after leptospiral infection, blood culturing and PCR were positive, whereas the MAT was negative.[43]

Serology

The most frequently used method for the serologic diagnosis of leptospirosis is the microscopic agglutination test (MAT).[11,14,45] For the MAT, dilutions of a dog's serum are incubated with suspensions of live leptospires and are then inspected by dark-field microscopy for agglutination. The highest dilution of serum causing 50% of the leptospires to agglutinate is reported as the titer.[15] Complicating MAT interpretation is the subjective effect of observer variation, even within the same laboratory. Because the MAT uses live leptospires as antigens, antigen standardization may not be consistent, and the live leptospires pose a biohazard to laboratory personnel.[15,45] Generally, a single titer > 1:800 in an unvaccinated dog with classic signs of canine leptospirosis is considered valid for a presumptive diagnosis of leptospirosis, although higher titers, ≥ 1:3200, are considered by some investigators to be a more reliable threshold for a positive test.[46] Because a single MAT may fail to detect antibodies in the early phase of disease or may give false-positive results after vaccination or exposure in enzootic areas, the authors of the Consensus Statement of the American College of Veterinary Internal Medicine (ACVIM) and of the European Consensus Statement on Leptospirosis in Dogs and Cats recommend paired serologic testing.[45–47] A 4-fold change in an MAT convalescent titer compared with the baseline titer is consistent with active infection.[2,45,47] Interpretation of the MAT is further complicated by the high-degree of cross-reaction that occurs between the different serogroups, especially in acute phase samples.[23] Dogs often have similar titers to all serovars of an individual serogroup and at the same time higher titers to a serogroup unrelated to the infecting serogroup.[14,15] This cross-reactivity in the acute phase, followed by relative serogroup-specificity in the convalescent phase, results from the detection in the MAT of both IgM and IgG antibodies (**Figure 3**) and the presence of several common antigens among leptospires.[15,47] Additionally, both the MAT and the ELISA test that detects canine IgG against serovars Icterohaemorrhagiae, Canicola, Pomona, and Grippotyphosa suffer from the same limitations; namely, they may not detect antibodies early in the course of infection but do detect their presence as a result of vaccination.[47] Despite widespread use of the MAT for the diagnosis of leptospirosis in dogs, investigators have noted that the test lacks the degree of sensitivity, specificity, and repeatability expected of a reference standard test.[44] These shortcomings have led to the development of rapid screening tests for detection of genus-specific IgM antibodies that become detectable during the first week of

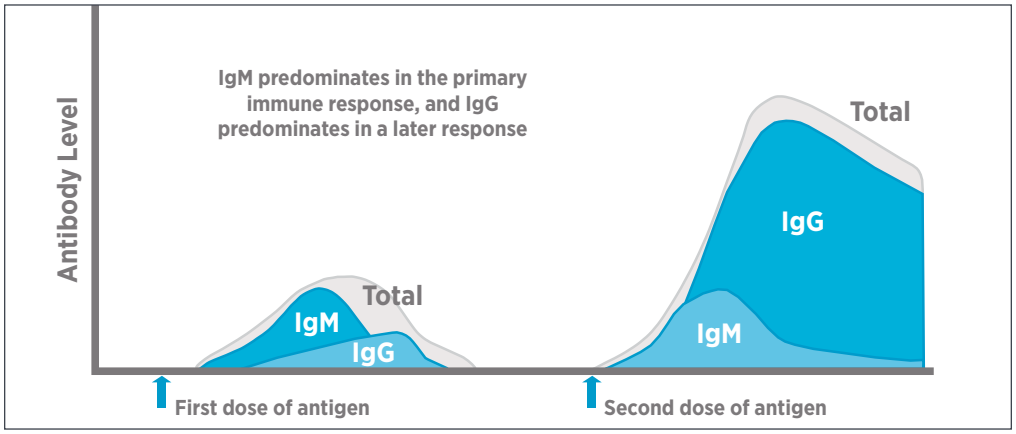


Figure 3. Relative amounts of IgM and IgG following the first and second exposure to antigen. Adapted from IR Tizard. *Veterinary Immunology—An Introduction*. 5th ed. Figure 135.

illness, allowing diagnosis to be confirmed and treatment to be initiated when it is most likely to be effective.[15]

Treatment and Prevention

The treatment recommended by the ACVIM Consensus Statement for optimal clearance of leptospiral organisms is doxycycline at 5 mg/kg by mouth every 12 hours for 14 days.[45] To prevent acute kidney damage, treatment should be started as soon as leptospirosis is suspected, even before confirmation of the diagnosis. If vomiting precludes administration of doxycycline, dogs with leptospirosis may be treated with ampicillin (20 mg/kg intravenously every 6 hours, with dose reduction for azotemic dogs) or penicillin G (25,000 to 40,000 U/kg intravenously every 12 hours).[45] Dogs should then receive doxycycline for 2 weeks after the gastrointestinal signs subside to eliminate leptospires from the renal tubules and help prevent a chronic carrier state.[2,45] Supportive therapy depends on severity of infection and degree of renal or hepatic dysfunction. Aggressive fluid therapy concurrent with the administration of antimicrobials is vital to the prevention and treatment of acute kidney damage.[2,16] With early diagnosis and treatment, the survival rate for dogs with leptospirosis ranges from 78% to 88%.[11,12,18]

Leptospirosis has been reported in all 50 states in the U.S., with higher numbers of infection noted in the Midwest, Northeast, and along the West Coast.[48] Increasingly, disease has been diagnosed in urban dogs with no apparent history of access to wildlife or environmental water sources, a phenomenon putatively explained by exposure to the urine of rodents or other wildlife that visit urban areas during the night.[47] For all at-risk dogs, the ACVIM and European Consensus Statements recommend vaccination with 4-serovar vaccines.[45,47] Canine infection with leptospires can also be diminished by limiting the contact of dogs with rodents and other reservoirs of disease and with other potential sources of infection, including contaminated marshy areas and standing water.[47]

SECTION 2.

WITNESS® AND RIM™

The first WITNESS® brand point of care diagnostic line utilizing Rapid Immuno Migration, or RIM™, technology was introduced in Europe in 1995. RIM™ is a test format that generally uses tagged colloidal gold particles as a color signal rather than an enzyme-catalyzed color change reaction as in ELISA. The WITNESS® Lepto test uses biologics and the colloidal gold system to allow detection of anti-*Leptospira* IgM antibodies in canine whole blood, serum, or plasma (**Figure 4**). The IgM antibodies directed against various leptospiral antigens become detectable during the first week of leptospirosis infections.[37]

Colloidal gold is used as the signal because its inherent stability allows the WITNESS® Lepto Test Kit to be stored without refrigeration for the duration of the kit's shelf life. The WITNESS® Lepto Test Kit provides point-of-care results in approximately 10 minutes.

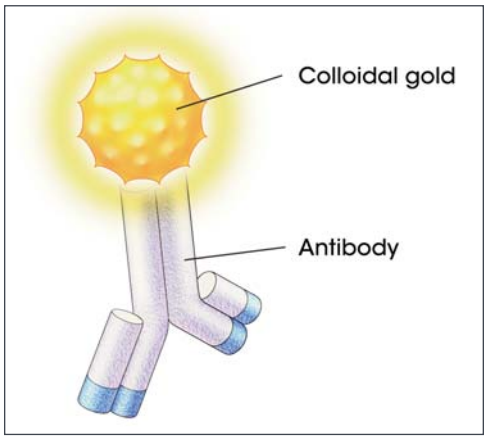


Figure 4. The WITNESS® Lepto test uses sensitized colloidal gold particles to form a complex with anti-*Leptospira* antibodies.

For the WITNESS® Lepto test, the colloidal gold is conjugated to goat antibodies targeting dog IgM.

The formed complexes migrate along the test strip. In dogs with circulating IgM antibodies to *Leptospira* spp., the complexes are then captured on a sensitized result line where their accumulation causes development of a pink to red color at the result line.

WITNESS® Lepto: How It Works

- 5 µL (0.005 mL) of sample (**Table 2**) is added directly to the sample well from the provided self-filling pipette by touching the tip of the pipette to the sample pad and carefully squeezing the pipette bulb (**Figure 5**). After the sample is absorbed, three drops (~140 µL) of buffer are added to the sample well.

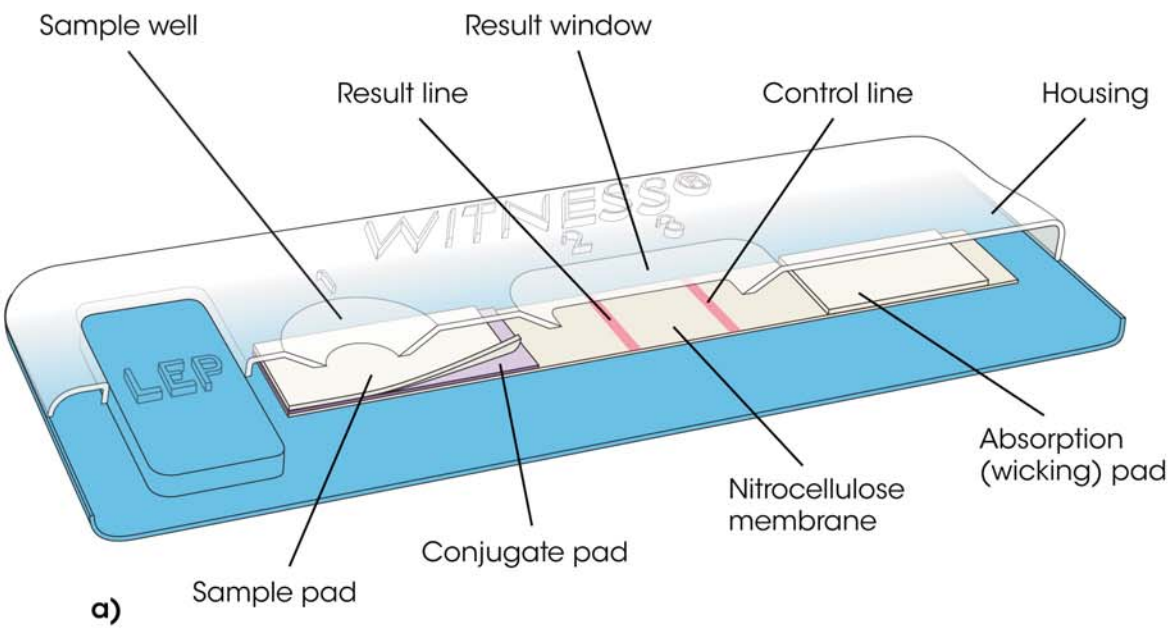
Table 2.

APPROPRIATE SAMPLE TYPES FOR THE WITNESS® LEPTO TEST

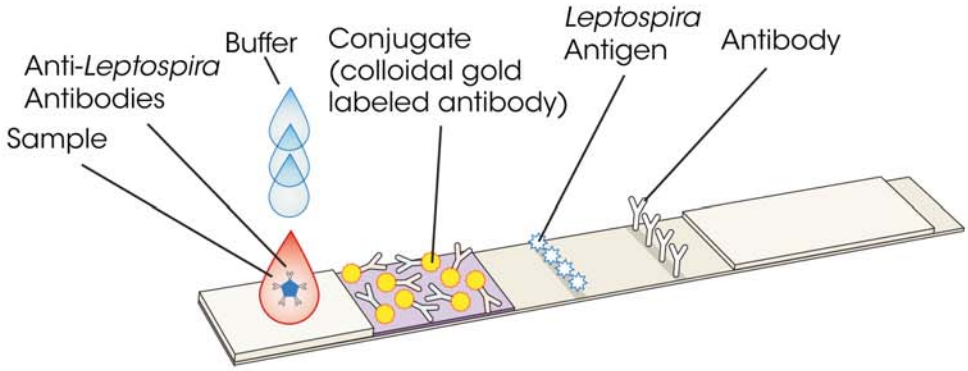
1. Anti-coagulated whole blood
2. Serum
3. Plasma

- The sample and buffer flow through the sample pad where blood cells and cellular debris are filtered, allowing the test sample to flow onto and across the conjugate pad.
- Sensitized colloidal gold particles form a complex with the IgM antibodies in the sample. The formed complexes continue migrating laterally across the nitrocellulose test membrane.
- The complexes are then captured on a result line composed of *Leptospira* antigens, where their accumulation causes the formation of a clearly visible pink to red line.
- Gold particles sensitized to an unrelated antigen continue to flow across the membrane towards the absorption (wicking) pad and are bound at the control line. A visible color line (pink to red) verifies that the test is working properly.

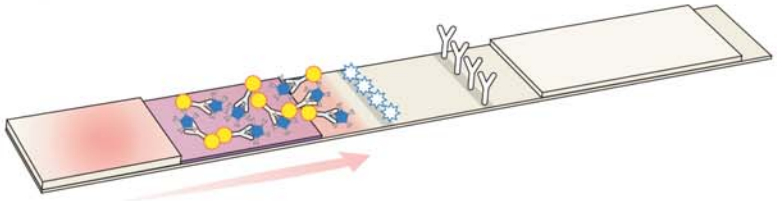
Introduced in Europe in 2015, the WITNESS® Lepto Test Kit has been thoroughly evaluated and has shown excellent sensitivity and specificity.[49]



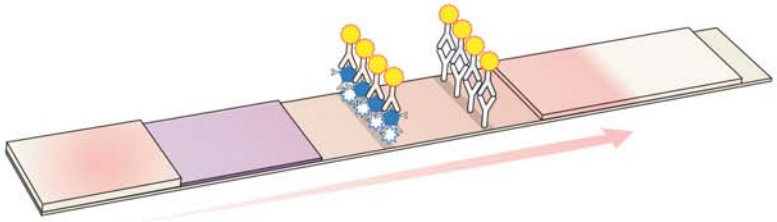
a)



b) *To initiate the test, one drop of sample and 3 drops of buffer are added to the sample well.*



c) *The sample/buffer and conjugate migrate across the nitrocellulose strip.*



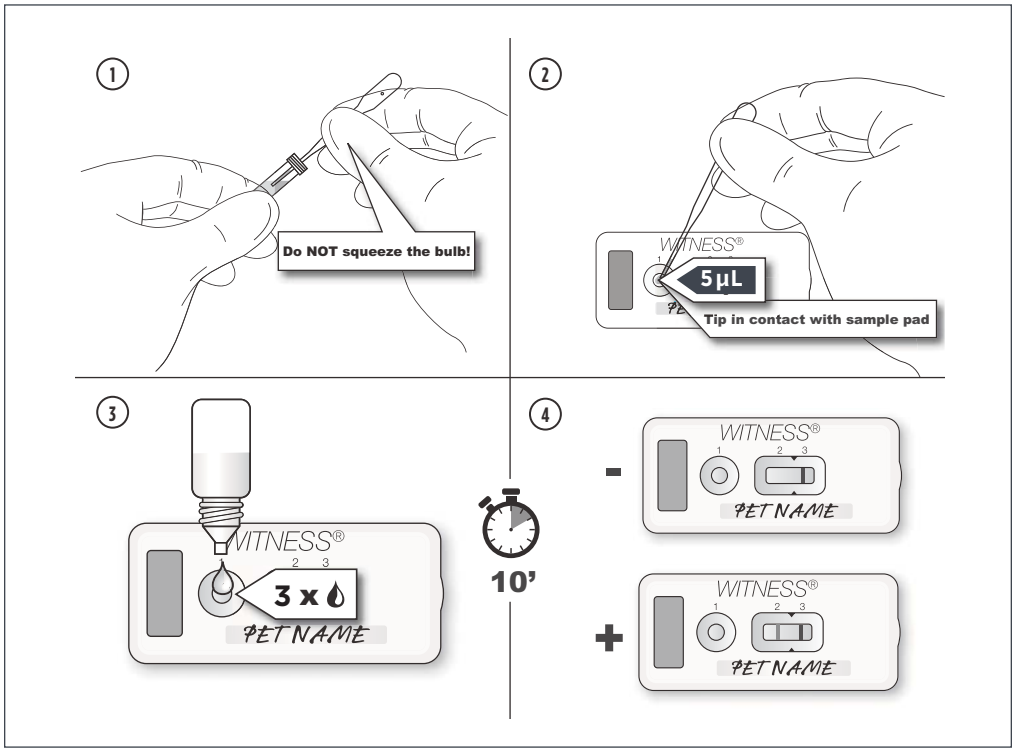
d) *Anti-Leptospira antibody bound conjugate is captured at the result line, and control line targeted conjugate is captured at the control line.*

Figure 5. WITNESS® Lepto Test Kit for dogs.

Instructions for Use

Below is a brief summary of how to use the WITNESS® Lepto Test Kit:

1. Grip the pipette by the stem. Hold a tube of sample and the pipette at an angle to allow proper filling of the pipette. **Without squeezing**, place the tip of the pipette in the sample. The capillary tube in the pipette will fill automatically in 2 to 3 seconds. Remove the pipette from the sample when the capillary tube has filled.
2. Touch the tip of the pipette to the sample pad and carefully squeeze the bulb to express the sample (5 µL), making sure fingers are covering the vent holes on the pipette bulb.
3. Add **3 drops** of buffer solution.
4. Read results in approximately 10 minutes.



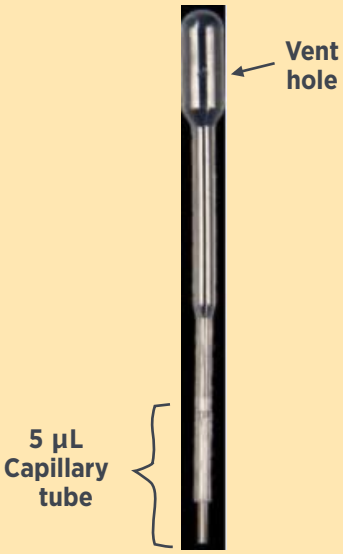
Guidelines for Optimizing Test Outcomes

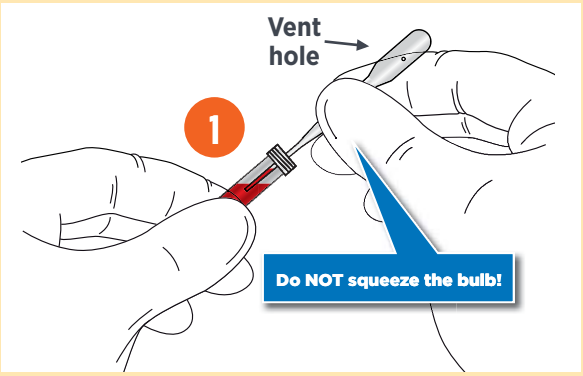
Various factors have the potential to affect the accuracy and performance of all diagnostic tests. Compliance with the guidelines below increases confidence that results obtained with the WITNESS® Lepto Test Kit are valid:

- **Always use the appropriate sample type specified in the test kit insert.** WITNESS® Lepto has been validated with anti-coagulated whole blood, serum, and plasma. Failure to use the appropriate sample type can result in erroneous results. A common error for whole blood sample type is not using anticoagulant or using an inappropriate ratio of anticoagulant to whole blood. This is often the result of the desire to apply blood directly from a syringe, which introduces another error, inadequate or excess sample volume. With too little or no anti-coagulant, the sample may not migrate; with too much, the sample may be diluted and therefore may yield false negative results. Sample volumes greater than the directed volume may result in a false positive test. Using syringes with or without the needle can adversely affect sample volume, absorption, migration, and test accuracy.

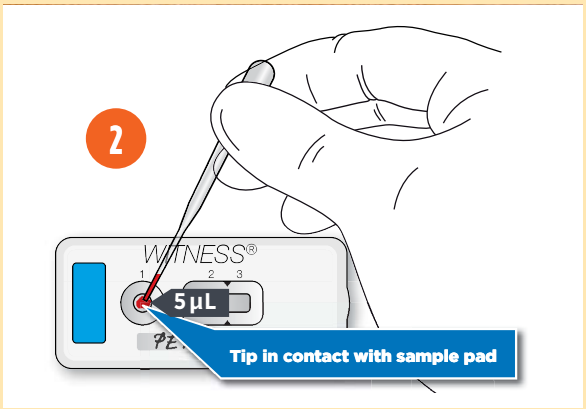
- **Always use test kit components before their expiration dates.**
- **Store test kits at label-specified temperatures.** WITNESS® tests are made to be stored without refrigeration (2°–25°C; 35°–77°F) and used at room temperature (20°–25°C; 68°–77°F). Do not freeze. If the test is refrigerated, it is recommended that the test be brought to room temperature prior to running.
- **Use the test kit immediately after opening the sealed pouch.** The WITNESS® Lepto test is sensitive to humidity and must be run within 10 minutes of its removal from the foil pouch.
- **IMPORTANT:** The WITNESS® Lepto test uses a special pipette (see below) that fills automatically and accurately by capillary action when placed in a sample. Do not squeeze to fill. Hold a tube of sample and the pipette at an angle to allow proper filling (see above right), which usually occurs in 2 to 3 seconds. Failure to follow instructions carefully may result in improper function of the device.

Special WITNESS® Lepto Pipette





- **Touch the tip of the pipette to the sample pad and carefully squeeze the bulb to express the sample (5 µL),** making sure fingers cover vent holes on the pipette bulb (see below). Too little of any sample type may lead to a weak positive or false negative result. Too much of any sample type may lead to a false positive result or sample leakage onto the membrane, making test results difficult to interpret (e.g., pink lines in Windows 2 and 3 may not be visible if anti-coagulated blood obscures them).



- **Use a separate pipette for each sample.**
- **Always hold the buffer bottle straight up and down when adding buffer (~140 µL) to the sample pad.** Holding the bottle at an angle affects the drop size and will result in an incorrect volume of buffer added to the test.
- **Place the WITNESS® cassette on a flat, horizontal surface while performing the test to ensure migration along the test strip.**

SECTION 3.

WITNESS® LEPTO SUPPORTING DATA[50]

Introduced in Europe in 1995, the WITNESS® brand point-of-care diagnostic line utilizing RIM™ technology has been found to be an easy to use test format having high sensitivity and specificity. In 2015, the WITNESS® platform was adapted to allow detection of anti-*Leptospira* IgM antibodies.

WITNESS® Lepto Test Usage:

- The test should be used in sick dogs with clinical signs compatible with canine leptospirosis: signs of renal and/or hepatic failures, lethargy, anorexia, vomiting, acute febrile illness, pulmonary hemorrhage, anemia, uveitis, abortion.
- WITNESS® Lepto detects the presence of IgM antibodies against *Leptospira* spp. in canine whole blood, plasma, or serum. The test is simple, requires minimal equipment and capabilities, and results are interpreted in approximately 10 minutes.

Summary Supporting Data[49,50]

Studies have been completed to validate the performance of the WITNESS® Lepto test kit.

WITNESS® Lepto Field Study Establishing Sensitivity and Specificity: In this study, 275 samples from dogs with clinical suspicion of acute leptospirosis were tested to determine sensitivity (ability to correctly identify a truly infected dog as infected) and specificity (ability to correctly identify an uninfected dog as *Leptospira*-negative) by comparing test results with those obtained with the reference microscopic agglutination test (MAT).

All 275 samples originated in the United States, with 216 samples from two university veterinary diagnostic laboratories (VDL A, n = 27; VDL B, n = 189), and 59 samples from 5 veterinary hospitals located in the states of Illinois or Michigan.

Results: In the WITNESS® Lepto Field Study evaluating the 275 samples from dogs where there was a clinical suspicion of acute leptospirosis, WITNESS® Lepto had a sensitivity of 83.7% (95% CI: 70.1%–91.8%) and a specificity of 90.2% (95% CI: 82.1%–94.8%) when compared to the MAT (**Table 3**).* Known challenges of MAT testing, including cross-reaction with vaccination, and the limitations of comparing to a single MAT sample have been well documented.[45,47] As such, the results shown for this study may underestimate the performance of the WITNESS® Lepto test (see below for additional details).

Table 3.
Meta-analysis results comparing WITNESS® Lepto and MAT*,†,‡,50

Sample Source	True Positive	True Negative	False Positive	False Negative	Sensitivity (Se) (95% CI)	Specificity (Sp) (95% CI)
Veterinary Hospital Submissions	6	50	2	1	**85.7%**	**96.2%**
VDL-A	5	21	1	0	**100%**	**95.5%**
VDL-B	66	94	15	14	**82.5%**	**86.2%**
WITNESS® Lepto vs MAT Meta-analysis‡	**77**	**165**	**18**	**15**	**83.7% (70.1%-91.8%)**	**90.2% (82.1%–94.8%)**

*Data has not been reviewed and approved by the USDA.
CI = confidence interval; MAT = microscopic agglutination test; VDL-A, VDL-B = samples from two university veterinary diagnostic laboratories.
†A sample was characterized as positive based upon MAT results and vaccination history, if known (positive samples had an MAT of ≥1:800 if vaccination history was not available, or ≥1:1600 if vaccinated > 3 months prior, or ≥1:3200 if vaccinated ≤ 3 months prior.)
‡A meta-analysis was performed because the field samples were comprised of subsets from three different populations.
Data on file, Study Report No. D860Z-US-028 Q005, Zoetis Inc.

Limitations of the MAT as a Serological Reference Test

In the field study evaluating sensitivity and specificity, results obtained with the WITNESS® Lepto test were compared with those obtained with the MAT. MAT testing is widely recognized as the serological reference standard for leptospirosis testing; however, interpreting test results can be problematic. This is an especially relevant consideration when paired serum samples (collecting two samples about 2 weeks apart looking for a four-fold increase in titers) and additional clinical context (history and clinical signs) are unavailable. MAT has several other limitations: it does not differentiate between antibodies produced through natural infection and those produced through vaccination; it is frequently associated with antibody cross-reaction between serovars; and it may not identify latent infections.[45,47,51]

For the field study summarized above, paired serum samples were not available, so the criteria in **Table 4** were used to define a positive case.

Table 4.
Field study data: diagnostic sensitivity and specificity calculations*,†,[50]

Sensitivity	83.7% (95% CI: 70.1%-91.8%)
Specificity	90.2% (95% CI: 82.1%–94.8%)
Number of samples	275
Reference	Sample considered POSITIVE if MAT:‡ ≥1:800 if no vaccination history, or ≥1:1600 if vaccinated > 3 months prior, or ≥1:3200 if vaccinated ≤ 3 months prior

*Data has not been reviewed and approved by the USDA.
†Meta-analysis field samples collected from veterinary hospitals (n = 59) and university veterinary diagnostic laboratories (n = 27 and n = 189).
‡Based on the serovar with the highest MAT titer of the serovars Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona (Serovar Bratislava was not included because it is not recognized as a pathogenic serovar in dogs in the U.S. by the USDA).
Data on file, Study No. D860Z-US-15-028 Q005, Zoetis Inc.

Additional Evaluations: **Table 5** (see page 14) contains summaries of additional studies to support the performance of WITNESS® Lepto.

Experimental Infection Studies:[54-57]
In these studies, four groups of male beagles (n = 8/group), approximately 6 months of age, were experimentally infected with *Leptospira interrogans* serovar Canicola, Icterohaemorrhagiae, Pomona, or *L. kirschneri* serovar Grippotyphosa. The dogs were challenged through intraocular, oral, and intranasal routes for three consecutive days. Sera were collected on study days 0, 4, 7, 10, 14, 17, 21, 24, and 28 for all serovars except for Grippotyphosa, in which sera was only collected to Day 14, and assayed on WITNESS® Lepto and MAT.

Results summarized in **Table 6** (see page 14) show that WITNESS® Lepto detected antibodies to *L. kirschneri* serovar Grippotyphosa, *L. interrogans* serovar Canicola, *L. interrogans* serovar Pomona, and *L. interrogans* serovar Ictero-haemorrhagiae challenge. Additionally, WITNESS® Lepto detected IgM in 8/8 dogs challenged with *L. kirschneri* sero-var Grippotyphosa, and *L. interrogans* serovvar Pomona by day 7 following challenge. WITNESS® Lepto detected antibody from all four serovars in all samples as early or earlier than MAT.

Table 5.
Synopses of additional studies[50,52,53]

Study	Study Design	Summary of Results
Analytical Specificity Evaluating test performance when dogs are infected with unrelated organisms or have similar clinical presentations	Leptospirosis negative dogs that were experimentally or naturally infected with *Borrelia borgdorferi* (n = 36), *Ehrlichia canis* (n = 27), Parvovirus (n = 8), Adenovirus (n = 10)	81/81 dogs negative (100% specificity).
Specificity in Healthy Dogs	Healthy purpose-bred laboratory dogs (n = 102); apparently healthy client-owned dogs (n = 50) from a veterinary hospital in Michigan (USA).	102/102 negative healthy purpose-bred laboratory dogs tested negative (100% specificity); 49/50 apparently healthy client-owned dogs tested negative (98% specificity).
Compatibility with Whole Blood	Anti-coagulated paired whole blood and serum or plasma samples were collected from three groups of dogs: 1) 30 samples spiked with positive sera; 2) 99 blood samples collected from purpose–bred laboratory dogs (naïve or previously enrolled in non-Lepto-related studies), 3) 24 samples from client-owned dogs with clinical signs consistent for leptospirosis.	1) 30/30 spiked (high/medium/low) samples tested positive. 2) 96/99 laboratory dogs negative (96.9% specificity). 3) 24/24 client-owned dogs had same result on the blood sample as the respective serum sample.

Table 6.
Comparison of WITNESS® Lepto and MAT for number dogs positive per day by *Leptospira* serogroup and serovar[52, 54-58]

Test	Serovar	Day 0	Day 4	Day 7	Day 10	Day 14	Day 17	Day 21	Day 24	Day 28
WITNESS® Lepto	Grippotyphosa*	0/8	0/8	8/8	8/8	6/6	3/3	NT	NT	NT
	Canicola*	0/8	0/8	8/8	8/8	4/4	4/4	4/4	4/4	4/4
	Pomona*	0/8	0/8	8/8	7/7	6/6	6/6	6/6	6/6	6/6
	Icterohaemorrhagiae	0/8	0/8	4/8	8/8	8/8	8/8	8/8	8/8	8/8
MAT	Grippotyphosa*	NT	0/8	6/8	7/8	5/6	3/3	NT	NT	NT
	Canicola*	0/8	0/8	2/8	7/8	4/4	4/4	4/4	1/4	4/4
	Pomona*	0/8	0/8	8/8	7/7	6/6	6/6	6/6	6/6	6/6
	Icterohaemorrhagiae	0/8	0/8	5/8	8/8	8/8	8/8	8/8	8/8	8/8

MAT = microscopic agglutination test; positivity is based on 4-fold seroconversion.
*Some dogs were removed from study early.

CONCLUSIONS

The epidemiology of canine leptospirosis has undergone significant changes since the 1970s when widespread administration of bivalent vaccines containing *Leptospira* serovars Canicola and Icterohaemorrhagiae greatly reduced the incidence of disease in Europe and North America.[11, 17–26] As urban development has spread into areas once considered rural, dogs increasingly have come into contact with *Leptospira* serovars shed by wildlife and livestock species. Since the 1990s, *Leptospira* serovars Pomona, Grippotyphosa, and Bratislava have increasingly been identified in infected dogs worldwide.[18,20,26–29] Emergence of the disease in household dogs caused by wildlife and livestock non-canine *Leptospira* serovars is particularly concerning because infection with non-canine adapted serovars is associated with clinical disease of greater severity and because infected dogs can pose a potential zoonotic risk to humans.[6,18,40]

Timely diagnosis of canine leptospirosis is vital because antibiotic therapy provides the greatest benefit when initiated early in the course of disease. Identification of early-phase leptospirosis is often delayed by the non-specific clinical presentation of the disease,[40] and laboratory methods available for direct detection of leptospires or their DNA—culture, dark field microscopy, and PCR—are time consuming, difficult to interpret, or not immediately available at the point of care.[1,2,5,14,15,37,43,44] Additionally, leptospirosis IgG tests are affected by vaccination.[47] The traditional serological test for diagnosis—MAT—can be difficult to perform and interpret, requires submission of samples to a reference laboratory, and is infrequently requisitioned in first-opinion practice.[2,11,14,15,23,44–47,59]

Introduced in Europe in 2015, the WITNESS® Lepto Test Kit uses Rapid Immuno Migration—RIM™—technology to detect the presence of IgM antibodies against *Leptospira* in three different sample types. The entire test can be completed at the point of care in approximately 10 minutes.

The studies described above demonstrate that WITNESS® Lepto has a high degree of sensitivity and specificity. In these studies:

- The test was positive with sera from all dogs experimentally infected with key serovars including *L. kirschneri* serovar Grippotyphosa, *L. interrogans* serovar Canicola, *L. interrogans* serovar Pomona, and *L. interrogans* serovar Ictero-haemorrhagiae.
- The test detected IgM seroconversion to experimental infection as early as, or earlier than, MAT in tested dogs.
- Test specificity was shown to be very high in both healthy dogs and dogs infected with diseases other than leptospirosis.
- The test was shown to be compatible with anti-coagulated whole blood samples as this sample matrix had minimal to no interference with yielding correct test results.

The test should be used in sick dogs with clinical signs compatible with canine leptospirosis: signs of renal and/or hepatic failures, lethargy, anorexia, vomiting, acute febrile illness, pulmonary hemorrhage, anemia, uveitis, and abortion. WITNESS® Lepto detects the presence of IgM antibodies against *Leptospira* in canine whole blood, plasma, or serum. The test is simple, requires minimal equipment and capabilities, and results are interpreted in approximately 10 minutes. Compatible clinical signs and a positive result on WITNESS® Lepto suggest acute leptospirosis.

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