IMMUNODOT™
LEPTOSPIRA IGM

IVD For In Vitro Diagnostic Use

CE

INTENDED USE

The GenBio IgM ImmunoDOT Leptospira test is a qualitative enzyme immunoassay that specifically detects IgM antibodies to *Leptospira biflexa* (serovar patoc 1). This test is presumptive for the laboratory diagnosis of leptospirosis. The IgM ImmunoDOT Test may be used to evaluate a single specimen or paired specimens to detect seroconversion. The test is intended for use in serum, plasma, heparinized whole blood or finger stick capillary blood.

SUMMARY AND EXPLANATION

Leptospirosis is a geographically widespread spirochetal disease that infects humans through contact of skin or mucous membranes with contaminated urine of wild or domestic animals. It is an acute febrile illness caused by members of the genus *Leptospira*, of which more than 200 serovars have been described in humans and in more than 180 species of animals in tropical areas of the world. Leptospirosis cases occur throughout the year, but approximately one half of infections occur from July to October.

Leptospires are obligate aerobes, appearing as motile, flexible, coiled rods of 6 to 20μm in length. The genus *Leptospira* includes the species *biflexa*, a free-living non-pathogenic spirochete found in water and soil samples. *L. biflexa* has been shown to have broad cross-reactivity among the species and has become the organism of choice for development of serological assays (1).

Leptospirosis may develop clinically as an asymptomatic anicteric influenza-like infection or a more severe hemorrhagic disease that may include meningitis, jaundice and renal failure. The latter icteric form was described by Adolph Weil in 1886, and became known as Weil's disease. The clinical manifestations of leptospirosis range from a mild catarrh-like illness to icteric disease with severe liver and kidney involvement. Natural reservoirs for leptospirosis include rodents as well as a large variety of domesticated mammals. The organisms occupy the lumen of nephritic tubules in their natural host and are shed into the urine. Human infection derives from direct exposure to infected animals. In the United States and elsewhere, occupational groups at particular risk for leptospirosis include persons employed in agriculture, sewer and construction workers as well as stock handlers. In urban areas contaminated well water, spring water and food preparation surfaces have been implicated in the transmission of disease to humans (2). Bathing or swimming in water sources where livestock have been pastured has been demonstrated to be a potential infection hazard. The organisms enter the host through skin abrasions, mucosal surfaces or the eye. The incubation period can range from 3 to 30 days but is usually found to be 10 to 12 days. IgM antibodies can be detected by the 6th to 10th day of disease and generally reach peak levels within 3 to 4 weeks. Antibody levels then gradually recede but may remain detectable for years. Epidemiologic factors, clinical findings, exposure in endemic regions, and other laboratory results should be considered in diagnosing acute disease. Acute disease diagnosis should include a laboratory confirmation.

The laboratory diagnosis of leptospirosis is based on serological methods. Although culture methods are understood to be of epidemiological importance, the time elapsed between the culture and the identification of the infecting organism permits only a retrospective diagnosis. Cultures from blood and cerebrospinal fluid grown on specific media during the first week of illness can be useful to confirm a diagnosis. However it may take 6-8 weeks for organisms to grow out (2).

The microscopic agglutination test (MAT) is the preferred method, and is the current World Health Organization standard reference method (3). The MAT method has high sensitivity and specificity and permits the detection of group-specific antibodies. The MAT test employs a battery of viable serovars, which are incubated with patient sera and viewed under a dark field microscope for agglutination. A variant of the procedure employs formalin treated organisms. However, fixed organisms are thought not to be as useful in detecting antibodies as live organisms (4). Unfortunately, the performance of the MAT test is limited to the few highly specialized laboratories in the world that are capable of maintaining live, hazardous stock serovar cultures.

Criteria for the laboratory diagnosis of leptospirosis have recently been established by the Centers for Disease Control and Prevention (CDC) (5). These criteria are the isolation of leptospira from a clinical specimen, or a fourfold or greater increase in leptospira agglutination titer between acute and convalescent-phase specimens obtained greater than or equal to two weeks apart, or the demonstration of leptospira in a clinical specimen by immunofluorescence. A presumptive diagnosis may be made on the basis of a leptospira agglutination titer of greater than or equal to 200 in single specimens from a clinically symptomatic case.

A number of methods have been described for the detection of IgM antibodies in acute phase sera, using enzyme-linked microplate immunosorbent (ELISA) assays. An IgM ELISA test for leptospirosis and an indirect hemagglutination (IHA) in vitro

diagnostic procedure are commercially available and are reported to have a sensitivity and specificity comparable to the MAT method (6). The MAT method has not been distributed commercially.

ASSAY PRINCIPLE

The GenBio IgM ImmunoDOT Leptospirosis Test utilizes an enzyme-linked immunoassay (EIA) dot technique for the detection of IgM antibodies. *L. biflexa,* serovar Patoc 1 strain antigens are dispensed as discrete dots onto a solid membrane.

After adding the test specimen to a reaction cuvette, an assay strip is inserted, allowing patient antibodies reactive with the test antigens to bind to the strip's solid support membrane. Alkaline phosphatase conjugated goat anti-human IgM antibodies are allowed to react with bound patient antibodies. Finally, the strip is transferred to an enzyme substrate reagent, which reacts with bound alkaline phosphatase to produce an easily seen, distinct spot.

REAGENTS

Assay Strips: Include a positive human IgM control, negative control and four dilutions of *L. biflexa* antigen

Diluent (#1): Consists of goat antihuman IgG, buffer salts with <0.1% NaN_3 (pH 6.2-7.6)

Enhancer (#2): Consists of sodium chloride with <0.1% NaN_3

Conjugate (#3): Consists of alkaline phosphatase conjugated goat anti-human IgM antibodies in buffered diluent (pH 6.2-7.6) with <0.1% NaN_3

Developer (#4): Consists of 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium chloride in buffered diluent (pH 9.0-11.0) with <0.1% NaN_3

Positive Control: Human anti–Leptospira serum containing <0.1% NaN_3

Negative Control: Nonreactive human serum containing <0.1% NaN_3

WARNINGS AND PRECAUTIONS

For In-Vitro Diagnostic Use. ImmunoDOT reagents have been optimized for use as a system. Do not substitute other manufacturers' reagents or other ImmunoDOT Assay System reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Do not use any kits beyond the stated expiration date. Analytic quality water must be used. Close adherence to the test procedure will assure optimal performance. Do not shorten or lengthen stated incubation times since these may result in poor assay performance.

Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. It may be harmful if enough is ingested (more than supplied in kit). On disposal of liquids, flush with a large volume of water to prevent azide build-up (7). This dilution is not subject to GHS, US HCS and EU Regulation 2008/1272/EC labeling requirements.

The safety data sheet (SDS) is available at support.genbio.com or upon request.

Human source material. Material used in the preparation of this product has been tested and found non-reactive for hepatitis B surface antigen (HBsAg), antibodies to hepatitis C virus (HCV), and antibodies to human immunodeficiency virus (HIV-1 and HIV-2). Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting infectious disease (8). Follow recommended Universal Precautions for bloodborne pathogens as defined by OSHA (9), Biosafety Level 2 guidelines from the current CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (10), WHO Laboratory Biosafety Manual (11), and/or local, regional and national regulations.

STORAGE

Store at 2-8°C. Reagents must be at room temperature (15-30°C) before use. Assuming good laboratory practices are used, opened reagents remain stable as indicated by the expiration date.

SPECIMEN COLLECTION AND HANDLING

The GenBio IgM ImmunoDOT leptospirosis test can be performed with serum, heparinized plasma, heparinized whole blood or finger stick capillary blood.

Do not use lipemic specimens.

Do not use specimens that are hemolyzed.

Do not use specimens that are bacterially contaminated.

The test requires approximately 10 μl of serum or plasma or 20 μl of whole blood. Serum, heparinized plasma, and heparinized whole blood should be collected according to standard practices. Heparinized whole blood may be stored for no longer than 8 hours at 22°C. Finger stick capillary blood should be used immediately. Freezing whole blood specimens is not recommended. It is recommended that acute and convalescent specimens obtained to determine seroconversion be collected equal to or greater than 2 weeks apart. Storage conditions for serum or plasma should be consistent with the NCCLS recommendation:
(Approved Standard – Procedures for the Handling and Processing of Blood Specimens, H18 A. 1990)

MATERIALS PROVIDED

Assay Strips   Conjugate (#3)
Diluent (#1)   Developer (#4)
Enhancer (#2)   Reaction Vessels
Positive Serum Control
Negative Serum Control

MATERIALS REQUIRED BUT NOT PROVIDED

Workstation   Timer
Pipets   Specimen collection apparatus (e.g., finger sticking device, venipuncture equipment)
Analytic quality water   Absorbent toweling to blot dry assay strips

SET-UP

1. Turn on Workstation and adjust to proper temperature if necessary. Refer to Workstation Instructions.
2. Remove 4 Reaction Vessels (per test) from the product box and insert into appropriate slots in Workstation. For the large Workstation, add water up to the fill line of the provided rinse container. For the small Workstation, use an appropriate container and sufficient water to cover all reactive windows of the assay strip.
3. Place 2 mL Diluent (#1) in Reaction Vessel #1; 2 mL Enhancer (#2) in Reaction Vessel #2; 2 mL Conjugate (#3) in Reaction Vessel #3; and 2 mL Developer (#4) in Reaction Vessel #4.
4. Wait ten minutes before beginning "Assay Procedure". During this time, specimen(s) may be added (step #5), Assay Strips labeled (step #6), and inserted into the Strip Holder (step #7).
5. Add patient specimen (approximately 10 µL serum or 20 µL of whole blood) to Reaction Vessel #1.
6. Appropriately label the Assay Strips.
7. If the large Workstation is used, insert the label end of the Assay Strip into the Strip Holder, one per groove, taking care not to touch the assay windows.

ASSAY PROCEDURE

1. Pre-wet Assay Strip by immersing in water Vessel for 30-60 seconds.
2. Using several (10-15) quick up and down motions with the Assay Strip, mix diluent and specimen thoroughly in RV #1. Let strip stand for 10 minutes in RV.
3. Remove Assay Strip from RV and swish in the water Vessel. Use a swift back and forth motion for 5-10 seconds allowing for optimal washing of the Assay Strip's membrane windows.
4. Place Assay Strip into RV #2. Mix thoroughly with several (5-10) quick up and down motions. Let strip stand for 5 minutes in RV.
5. Remove Assay Strip from RV #2 and swish in the water Vessel as described (step 3).
6. Place Assay Strip into RV #3. Mix thoroughly with several (5-10) quick up and down motions. Let strip stand in RV #3 for 15 minutes.
7. Remove Assay Strip from RV #3 and swish in the water Vessel as described (step 3). DO NOT remove Assay Strip from the water Vessel.
8. Allow the Assay Strip to stand in the water Vessel for 5 minutes.
9. Remove Assay Strip from water Vessel and place into RV #4. Mix thoroughly with several (5-10) quick up and down motions. Let strip stand for 5 minutes in RV.
10. Remove Assay Strip from RV #4 and swish in the water Vessel as described (step 3).
11. Blot and allow Assay Strip to dry. It is imperative that tests of borderline specimens be interpreted after the Assay Strip has been allowed to dry.

QUALITY CONTROL

The top two membrane windows of the Assay Strip contain reagent controls. The dot in the top window is a positive reagent control containing human IgM and must be positive for further interpretation. This control indicates that the anti-human IgM conjugate is working properly. The dot in the second window is the negative antigen control (diluent and non-reactive proteins) and must be negative (white to pale-gray) for further interpretation. Reagent controls are intended to assure that reagents are active, and that the test has been performed properly.

If either reagent control is invalid, the test results should not be reported, and the test repeated. The intensity of the positive reagent control reaction may not be used as a guide to determine the intensity of dot-blots. Positive reactions in the other antigen windows of the strip may be either darker or lighter than the positive reagent control depending on the antibody titer. A weakly positive control serum and negative control serum are provided in the kit. The purpose of the weak positive control serum is to validate the assay near the cutoff point (2.0-2.5 dots). The performance of each test run should be confirmed by

performing a determination using the weakly positive control serum and obtaining a result of 2.0-2.5 dots. The test run should be repeated if a result of 2.0-2.5 dots is not obtained with the weakly positive control serum.

The assay's reagent temperature is between 42-48°C. Due to heat transfer loss, the Workstation temperature is set higher. The appropriate Workstation temperature setting is listed in the Workstation's package insert. (Contact Technical Services for additional guidance if an alternate heat source is used.)
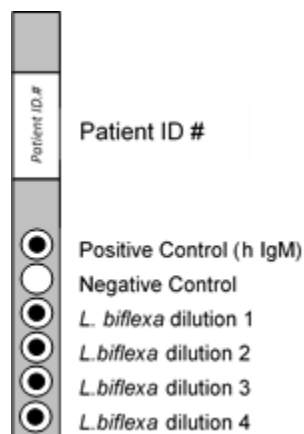
INTERPRETATION

Positive:	A dot with an easily seen, distinct border is visible in the center of the window. The outer perimeter of the window must be a white to pale gray color.
Intermediate	Intermediate reactions (those in which the intensity of the dot is greater or less than a whole dot) may be recorded as "0.5". For example, a positive test with a reactivity that is greater than 2.0 but less than 3.0 dots, or greater than 3.0 but less than 4.0 dots may be recorded as a 2.5 or 3.5 dot reaction, respectively.
Negative	If no dot is seen or a dot is difficult to see, interpret it as negative. Results are interpreted as the number of reactive *L. biflexa* dots: 0 to 1 for negative and 2-2.5 for borderline positive and 3-4 for a strong positive. Negative results do not rule out the diagnosis of leptospirosis. The specimen may have been drawn before the appearance of detectable antibodies. Negative results in suspected early leptospirosis disease should be repeated with a new specimen collected 2 weeks later.

Specimens showing a borderline positive reaction (2.0-2.5 dots) should be further tested with an additional specimen or an alternate test method. It is recommended that a convalescent specimen be collected from patients showing either an initially non-reactive (less than 2.0 dot) or borderline positive (2.0-2.5 dot) result.

Single specimens are used to assess exposure; paired specimens collected at different times from the same individual are required to demonstrate seroconversion. Paired specimens should be tested at the same time.

Seroconversion should be interpreted as the conversion from a negative first specimen to a positive second specimen when paired specimens are analyzed at least 2 weeks apart. Second specimens should show a reaction that is greater than borderline (2.0-2.5 dots). Second specimens not showing a reaction greater than borderline should be further tested with an additional specimen or an alternate test method to establish seroconversion.

Because the assay is qualitative, the magnitude of the measured result above the cutoff is not indicative of the total amount of antibody present.

Initially non-reactive: Samples interpreted as non-reactive (no reactive dots) indicate that antibody is not present in the sample, or is below the detection level of the method. Since antibodies may not be present during early disease, confirmation 2-3 weeks later is recommended. An initially negative result followed by a positive result indicates IgM seroconversion.

Borderline reactive: Borderline positive specimens (2.0-2.5 dots) should be cautiously interpreted. These specimens should be further tested with an additional specimen. If the specimen remains borderline reactive, a second serological method should be considered if leptospirosis infection is still suspected.

Initially reactive: Samples interpreted as strongly reactive (3 or 4 reactive dots) may indicate the presence of specific antibody. Antibody presence alone cannot be used for diagnosis of acute infection, however, because antibodies from prior exposure may circulate for a prolonged period of time.

LIMITATIONS

- Treatment is often indicated prior to completion of serologic diagnosis, which requires at least two weeks. Diagnosis of these diseases should not be made based on results of the IgM ImmunoDOT leptospirosis test alone, but in conjunction with other clinical signs and symptoms and other laboratory findings.
- Epidemiologic factors, clinical findings, exposure in endemic regions, and other laboratory results should be considered when making a diagnosis. Since serological assay methods may yield different results for weakly reactive specimens, a second serological method is recommended when the suspicion of infection remains. (See Interpretation of the Test, above).
- The temporal IgM response may be quite variable among leptospirosis patients. The IgM ImmunoDOT test may not be used to predict the point of IgM appearance or cessation in individual patients. This test may not distinguish a current or recent leptospirosis infection from a past infection or reinfection with another serotype.
- A leptospirosis infection cannot be ruled out by a negative IgM ImmunoDOT test or any other serological method. A negative specimen from a symptomatic patient indicates the need for analysis of a second specimen or alternative test methodology.
- A study of the capacity of the Diluent to deplete IgG from samples prior to IgM measurement indicated that more than 80.5% of IgG was absorbed. Additional goat antihuman IgG may be required if test specimens are suspected of abnormally high levels of IgG.
- Some high hematocrit samples (greater than 49.5%) may have lower reactivities with the test.
- Studies have indicated that 7.5% (55/731) specimens from symptomatic patients showed a 2.0 dot cutoff value with the IgM ImmunoDOT test. Twenty two of these (40%) were MAT positive and 9 (16.4%) were IHA positive at the cutoff. Therefore, the 2.0 dot reactive acute-phase specimens could be interpreted as false positives and the analysis of a second specimen collected 2-3 weeks later or use of an alternative test methodology is highly recommended.
- The potential effects on the performance of the test from interfering substances including lipids, bacterial contamination of the specimen, bile, or sample hemolysis have not been determined.
- The specificity study indicated that 20% of the Salmonella typhi reactive specimens were positive in the ImmunoDOT *L. biflexa* test. Therefore, the test may be cross-reactive with this organism.
- Antibody titers to leptospirosis may be delayed or substantially decreased by early and intensive antibiotic treatment.

EXPECTED RESULTS

Two independent studies, one in a rural and the other in an urban area of Maryland, were conducted with samples from asymptomatic normal donors. In the first study, sera were collected from 216 donors during the period of May through October of 1997. These rural samples were from the Maryland Eastern Shore with no known prevalence of leptospirosis. Sera from 207 of these donors were analyzed with the IgM ImmunoDOT leptospirosis test. Two sera (1.0%) were positive and 205 (99.0%) were negative. In the second study of 100 urban samples from normal donors, 97 were negative with the IgM ImmunoDOT leptospirosis test and 3 were positive. Using other leptospirosis assay methods, the frequency of positive specimens among normal donors was reported to be 0.1% in Hawaii and less than 0.001% in the continental United States (12).

PERFORMANCE CHARACTERISTICS

ASSAY CUTOFF ANALYSIS

The Receiver Operating Characteristic (ROC) dot-plot analysis (13) was used to examine the IgM ImmunoDOT test distribution of reactivity and cutoff points for acute and convalescent phase leptospirosis and non-leptospirosis specimens analyzed in Study No. 3. The ROC plot is shown in Figure 1.

Figure 1

Receiver Operator Characteristics (ROC) Plot
Leptopsirosis and Non-leptospirosis Patients
in Portugal
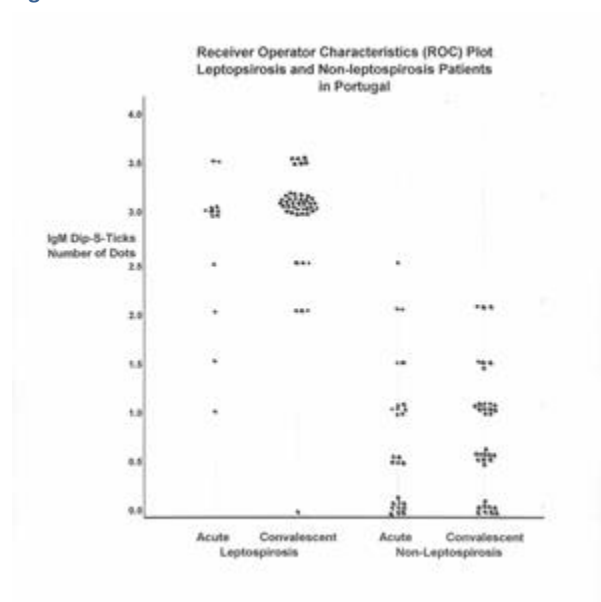
ACUTE-PHASE SPECIMEN

Acute-phase specimens were those obtained <10 days post-onset. Convalescent-phase specimens were those obtained 10-30 days and >30 days post-onset. Among 58 leptospirosis specimens, 13 were acute-phase and 45 were convalescent-phase. The percent positive agreement with the MAT test was 84.6% among acute-phase specimens and 97.8% among convalescent-phase specimens. Among 64 non-leptospirosis specimens, 27 were acute-phase and 37 were convalescent-phase. The percent negative agreement with the MAT test was 88.9% among acute-phase specimens and 91.9% among convalescent-phase specimens.

Since relatively few patients showed IgM ImmunoDOT values at the 2.0 dot cutoff point in the above study, the performance of the test at the cutoff point for all clinical study sites, as outlined in above, was determined. In this analysis, only 7.5% (55/731) specimens from symptomatic patients showed a 2.0 dot cutoff value with the IgM ImmunoDOT test. Twenty-two of these (40%) were MAT positive and 9 (16.4%) were IHA positive at the cutoff. Therefore, the 2.0 dot reactive acute-phase specimens could be interpreted as false positives and the analysis of a second specimen collected 2-3 weeks later or use of an alternative test methodology is highly recommended.

REPRODUCIBILITY (PRECISION) STUDY

This study was intended to assess the inter-laboratory reproducibility of the IgM ImmunoDOT Leptospirosis test. A panel of 30 coded and blinded leptospirosis-positive and negative sera was analyzed in each of three laboratories, including two clinical laboratories and the manufacturer's location. Positive sera were selected in a manner to represent different time periods of leptospirosis IgM antibody reactivity. Ten sera analyzed in each laboratory were obtained from patients less than ten days post-onset of infection and ten of the sera were obtained 10 to 30 days post-onset. In addition, ten of the sera were from negative controls.

To enable the demonstration of reproducibility throughout the entire measurement range of the IgM ImmunoDOT Leptospira test, the 15 leptospirosis-positive sera included samples in the reactivity range of 2.0 to 3.0 dots and the reactivity range of 3.0 to 4/0 dots. The ten negative sera were in the range of less than 2.0 dots, or less than the cutoff point for a positive test. Table 1 describes the reproducibility study of positive samples in three independent laboratories. Negative samples were consistently non-reactive in all laboratories.

Table 1: Leptospirosis Positive Samples

Sample	Days Post Onset	Reproducibility Data (Number of Positive Dots)			Mean	1 SD
		Site #1	Site #2	Site #3		
37094	10	2.5	2.5	2.5	2.50	0.00
37195	10	4.0	4.0	4.0	4.00	0.00
37357	10	4.0	3.5	3.5	3.83	0.29
37414	10	4.0	3.5	3.0	3.50	0.50
36404	10	3.0	2.5	3.0	2.83	0.29

Sample	Days Post Onset	Reproducibility Data (Number of Positive Dots)			Mean	1 SD
		Site #1	Site #2	Site #3		
36049	10	2.5	2.5	2.5	2.50	0.00
36308	10	4.0	4.0	4.0	4.00	0.00
37103	10	4.0	3.5	3.5	3.67	0.29
37655	10	4.0	3.5	3.0	3.50	0.50
29341	10	3.0	2.5	3.0	2.83	0.29
36243	10 - 30	4.0	4.0	3.5	3.83	0.29
36291	10 - 30	4.0	4.0	4.0	4.00	0.00
38476	10 - 30	4.0	4.0	4.0	4.00	0.00
36624	10 - 30	4.0	4.0	4.0	4.00	0.00
37064	10 - 30	3.5	4.0	3.5	3.66	0.29
37085	10 - 30	4.0	4.0	4.0	4.00	0.00
37195	10 - 30	4.0	4.0	4.0	4.00	0.00
37374	10 - 30	4.0	4.0	4.0	4.00	0.00
37440	10 - 30	4.0	3.5	3.0	3.50	0.50
37521	10 - 30	4.0	4.0	3.5	3.83	0.29
Mean		**3.7**	**3.6**	**3.5**		
1 SD		**0.53**	**0.59**	**0.53**		

A second study of the reproducibility of the IgM ImmunoDOT test was performed. Ten masked clinical specimens were each analyzed in triplicate (n=30) for 3 test days at each of three clinical sites. The mean number of dots, standard deviation (S.D.) and coefficient of variation (CV) were determined for each specimen in each site on each test day, as shown in Table 2, Table 3 and Table 4.

Table 2: Reproducibility of IgM ImmunoDOT TEST (Site 1)

Test Days	Mean # dots	S.D.	CV (%)	N
1	2.4	0.20	8.48	10
2	2.4	0.24	10.09	10
3	2.5	0.21	8.33	10
Total	**2.4**	**0.22**	**8.97**	**30**

Table 3: Reproducibility of IgM ImmunoDOT TEST (Site 2)

Test Days	Mean # dots	S.D.	CV (%)	N
1	2.4	0.17	7.10	10
2	2.4	0.23	9.92	10
3	2.5	0.18	7.21	10
Total	**2.4**	**0.20**	**8.08**	**30**

Table 4: Reproducibility of IgM ImmunoDOT TEST (Site 3)

Test Days	Mean # dots	S.D.	CV (%)	N
1	2.5	0.28	11.05	10
2	2.5	0.25	9.74	10
3	2.7	0.35	13.25	10
Total	**2.6**	**0.29**	**11.34**	**30**

The reproducibility of the controls provided in the test kit was also evaluated, by the analysis of 2 positive and 1 negative control samples for 10 days at each of 3 clinical sites. These data indicated a very high level of day-to-day and within-day reproducibility among the study sites.

SPECIFICITY (CROSS-REACTIVITY) STUDY

This study consisted of a panel of 170 specimens from patients with diseases other than leptospirosis. Samples were selected to represent infectious and non-infectious diseases having a febrile phase that may clinically mimic or be confused with leptospirosis. The numbers of each specimen type tested are indicated in parentheses.

These were parasitic and bacterial diseases (42) including (syphilis (11) scrub typhus (10) dengue (9) mycoplasma (8), tularensis (2) , and toxoplasma (2). Also included were ANA positive autoimmune (35), rickettsia (9), ehrlichia (3), Lyme disease (5), *Salmonella typhi* (10), Chagas (4) and miscellaneous (5). Viral diseases (57) included CMV (11), EBV (12), hepatitis (7), HIV (2), HTLV (1), HSV (10) rubeola and rubella (3) and VZV (11). Results of the study indicated that 163 specimens (95.9%) were negative and 7 (4.1%) were false positive when analyzed with the IgM ImmunoDOT test. Among false positive specimens were 1 each of ANA, HSV, syphilis, scrub typhus, dengue and 2 of *Salmonella typhi*. All were borderline positive, including 5 of 2.0 dots, and 2 of 2.5 dots.

CLINICAL STUDIES

CORRELATION (COMPARISON) STUDIES

Three independent studies compared the IgM ImmunoDOT leptospirosis test with other serological methods. In these studies, the Microscopic Agglutination Test (MAT) was considered as the reference method and primary basis for comparison. The commercially available Indirect Hemagglutination (IHA) Test, although intended for the detection of both IgM and IgG antibodies, was also employed.

Paired acute and convalescent specimens, as well as single specimens, from patients having clinical symptoms consistent with leptospirosis were analyzed in all studies. Test comparisons we based on the expression of the number of true positive (TP), true negative (TN), false positive (FP and false negative (FN) scores on individual specimens, assuming that the comparison methods (MAT and IHA) represent the TP result. The relative sensitivity, relative specificity and 95% confidence intervals of the sensitivity and specificity of the IgM ImmunoDOT test was determined for each study. Studies in which the MAT test was performed were identified as disease-confirmed or presumptive, based on the Centers for Disease Control and Prevention (CDC) case definition for leptospirosis (5). These criteria identify disease-confirmed patients as having a fourfold or greater increase in antibody titer between acute and convalescent-phase specimens obtained equal to or greater than 2 weeks apart. Presumptive patients lack a fourfold increase in antibody titer but show a titer of equal to or greater than 200 in one more specimens.

The cutoff points for a positive test for each of the methods employed are consistent throughout the studies, and are as follows:

- The IgM ImmunoDOT test is positive with a number of dipstick dots equal to or greater than 2.0.
- The MAT test is scored positive with a reciprocal serum titer equal to or greater than 1:160 to 200, depending on variations in the manner that the test is conducted in the relatively few specialized laboratories performing this procedure. The MAT score is based on the highest reciprocal titer of any 1 of 16 Leptospira strains analyzed in both the acute or convalescent-phase responses.
- The IHA test is scored as positive with reactivity equal to or greater than 1 at a 1:50 (screening) serum dilution.

A domestic prospective study (Study No. 1) of single and paired specimens from 286 symptomatic patients was conducted at the Hawaii Department of Health, Honolulu, Hawaii. The MAT, IHA and IgM ImmunoDOT tests were employed in the analysis of all specimens (Table 5).

Paired specimens were obtained from 241 patients in this study. Twenty-four patients seroconverted from negative to positive by the MAT test. Of these, 16 (69.6%) also seroconverted by the IgM ImmunoDOT test. Thirty patients (12.4%) were confirmed by the MAT method, with a 4-fold increase in antibody titer. Twenty-five patients (10.4%) were scored as presumptive by the MAT; presumptive patients lacked a 4-fold increase in MAT titer, but had titers > 160 in the convalescent specimen.

An analysis of the performance of the commercially available IHA test was also conducted among MAT confirmed patients and among MAT presumptive patients. The performance of the IHA test on MAT confirmed patients showed 19 of 30 (63.3%) patients positive with the IHA test. Eleven of 30 (36.7%) patients showing a 4-fold increase in MAT titer were IHA negative in both first and second specimens.

Among the 25 MAT-presumptive patients, the IHA test was positive in only 4 (16.0%).

The comparison analysis of methods was based on the number of days post-onset that individual specimens were obtained from symptomatic patients. Table 5summarizes specimens obtained less than 10 days, 10-30 days, greater than 30 days post-onset and all data. The number of true positive (TP), true negative (TN), false positive (FP) false negative (FN), relative sensitivity, relative specificity and 95% confidence intervals of the sensitivity and specificity are included for each group.

Table 5: Comparison of Methods Prospective Study in Hawaii

Methods Compared	Days Post Onset	TP	TN	FP	FN	Total	Relative Sensitivity (%)	Confidence Interval (95%)	Relative Specificity (%)	Confidence Interval (95%)
IHA and	All Data*	29	467	39	2	537	93.5	78.6-99.2	92.3	89.6-94.5
IgM ImmunoDOT	<10 Days	3	182	17	0	202	100.0	29.2-100.0	91.5	86.7-94.9
	10-30 Days	20	129	11	2	162	90.0	70.8-98.9	92.1	86.4-96.0
	>30 Days	6	156	11	0	173	100.0	54.1-100.0	93.4	88.5-96.7
MAT and	All Data*	53	442	21	29	545	64.6	53.3-74.9	95.5	93.1-97.2
IgM ImmunoDOT	<10 Days	11	171	10	10	202	52.4	29.8-74.3	94.5	90.1-97.3
	10-30 Days	26	120	9	9	164	74.3	56.7-87.5	93.0	87.2-96.8
	>30 Days	16	148	3	9	176	64.0	42.5-82.0	98.0	94.3-99.6
IHA and MAT	All Data*	30	458	1	49	538	38.0	27.3-49.6	99.8	98.8-100.0
	<10 Days	3	179	0	20	202	13.0	10.6-15.4	100.0	98.0-100.0
	10-30 Days	20	128	1	11	160	64.5	45.4-80.8	99.2	95.8-100.0
	>30 Days	6	150	0	18	174	25.0	10.7-50.2	100.0	97.6-100.0

*Total for "All Data" may exceed subtotals for the number of days post-onset since this information was unavailable for some patients.

A retrospective study (Study No. 2) was conducted in Barbados, consisting of paired specimens from 51 symptomatic leptospirosis patients and paired specimens from 52 symptomatic patients diagnosed with febrile diseases other than leptospirosis (Table 6).

The analysis of leptospirosis-confirmed patients indicated that a total of 40 of 51 (78.4%) showed a 4-fold increase in MAT antibody titer. Forty nine of 51 patients showed positive MAT titers in both the first and second specimens, and thus the true rate of seroconversion could not be determined.

Among presumptive patients, 9 of 51 (17.6%) of patients were scored as presumptive by the MAT test. Eight of these showed extremely high first specimen titers (6400-51,200), precluding a 4-fold increase in titer. All of these were positive in the IgM ImmunoDOT test in both the first and second specimens. All of these were also positive by the IHA test, except for 1 patient not IHA tested. These results indicated good agreement between all test methods.

Table 6 summarizes specimens obtained less than 10 days, 10-30 days post-onset and all data. In this study, no specimens were obtained greater than 30 days post-onset. The number of true positive (TP), true negative (TN), false positive (FP) false negative (FN), relative sensitivity, relative specificity and 95% confidence intervals of the sensitivity and specificity are included for each group.

Table 6: Comparison of methods retrospective study in Barbados

Methods Compared	Days Post Onset	TP	TN	FP	FN	Total	Relative Sensitivity (%)	Confidence Interval (95%)	Relative Specificity (%)	Confidence Interval (95%)
IHA and	All Data*	70	105	18	4	197	94.6	86.7-98.5	85.4	79.1-91.6
IgM ImmunoDOT	<10 Days	34	61	16	1	112	97.1	85.1-99.9	80.3	69.5-88.5
	10-30 Days	24	26	2	2	54	92.3	74.9-99.1	92.9	76.5-99.1
MAT and	All Data*	72	109	17	7	205	91.1	82.6-96.4	86.5	80.5-92.5
IgM ImmunoDOT	<10 Days	37	62	14	3	116	92.5	79.6-98.4	81.6	71.0-89.5
	10-30 Days	25	28	2	3	58	89.3	71.8-97.7	93.3	77.9-99.2
IHA and MAT	All Data*	62	110	10	15	197	80.5	69.9-88.7	91.7	85.2-95.9
	<10 Days	27	66	7	12	112	69.2	52.4-83.0	90.4	81.2-96.1
	10-30 Days	24	25	2	3	54	88.9	70.8-97.7	92.6	75.7-99.1

*Totals for "All Data" may exceed subtotals for the number of days post-onset since this information was unavailable for some patients.

A retrospective study (Study No. 3) was conducted at the Institute for Hygiene and Tropical Medicine, Lisbon, Portugal. This study consisted of 69 paired specimens from symptomatic patients comparing the performance of the MAT and IgM ImmunoDOT tests. These data showed relatively few leptospirosis-confirmed patients. Forty patients were scored as presumptive with a MAT reciprocal titer greater than or equal to 200, including 11 with a MAT-positive first specimen and 29 with MAT-positive first and second specimens. Eleven patients showed seroconversion from negative to positive by the MAT test. Of these, 6 (54.5%) also seroconverted with the IgM ImmunoDOT test.

Table 7 shows specimens obtained less than 10 days, 10-30 days and greater than 30 days post-onset as well as all data obtained. The number of true positive (TP), true negative (TN), false positive (FP) false negative (FN), relative sensitivity, relative specificity and 95% confidence intervals of the sensitivity and specificity are included for each group.

Table 7: Comparison of methods retrospective study in Portugal

Methods Compared	Days Post Onset	TP	TN	FP	FN	Total	Relative Sensitivity (%)	Confidence Interval (95%)	Relative Specificity (%)	Confidence Interval (95%)
MAT and	All Data*	65	63	7	2	137	97.0	89.6-99.6	90.0	80.5-95.9
IgM ImmunoDOT	<10 Days	11	24	3	2	40	84.6	54.5-98.1	88.9	70.8-97.7
	10-30 Days	29	19	1	0	49	100.0	88.1-100.0	95.0	75.1-99.9
	>30 Days	15	15	2	1	33	93.8	69.8-99.8	88.2	63.6-98.5

*Totals for "All Data" may exceed subtotals for the number of days post-onset since this information was unavailable for some patients.

Note: "Relative" refers to the comparison of this assay's results to that of a similar assay. There was not an attempt to correlate the assay's results with disease presence or absence. No judgment can be made on the comparison assay's accuracy to predict disease.

Conclusions of Correlation- Studies

The performance of the IgM ImmunoDOT test was evaluated by comparison with the commercially available IHA test and MAT reference test in three independent studies. The studies varied considerably in the number of those who could be leptospirosis-confirmed by a 4-fold increase in MAT antibody titer, and many patients, including those with high IgM antibody titers, remained only presumptive.

The results of the studies indicate a good relative sensitivity and specificity for the IgM ImmunoDOT leptospirosis test when compared to the IHA and MAT tests. However, Study No. 1 showed a much better relative sensitivity for the comparison of IHA and IgM ImmunoDOT tests than for the comparison of the MAT and IgM ImmunoDOT and the MAT and IHA tests. A good relative specificity was also reported in these studies.

TIME-COURSE STUDY

This study of serial specimens of leptospirosis-confirmed patients was also conducted in Barbados. The purpose of the study was to compare the relative antibody reactivities of the tests and to define the time of earliest leptospirosis antibody detection post-onset and during an additional period of time that included both the acute and convalescent phases of disease. It was also of interest to compare the overall antibody reactivity profile of the tests, including the point of declining antibody response during the late convalescent period. The antibody reactivities of the IgM ImmunoDOT, MAT and IHA tests were determined on serial bleed dates for each of 4 leptospirosis-confirmed patients. This study is shown in Table 8, Table 9, Table 10 and Table 11. These data from patient RB indicates that the MAT test was negative on day 4 post-onset while other tests were at least minimally positive. All tests were positive by day 10 and the IgM ImmunoDOT and IHA tests were strongly positive. All tests except IHA showed declining titers by day 67. In patient 1016, all tests were negative on day 2 but positive on day 6 post-onset. In patient 1259, all tests showed substantial titers on day 5 post-onset and remained so throughout the time-course of 62 days. Positive responses in all tests on day 1 in patient 1386 suggest the presence of IgM antibody from a prior leptospirosis infection. These data indicate that the temporal IgM response may be quite variable among leptospirosis patients and that the tests may not differentiate a current or recent infection from a past infection.

Table 8: Time-Course (Kinetic) Study Patients in Barbados: Patient RB

Test Types	Days Post Onset				
	4	10	16	24	67
MAT Titer	0	800	3200	6400	400
IgM ImmunoDOT positive dots	2.5	4.0	4.0	4.0	3.0
IHA Relative Titer	1.0	4.0	4.0	4.0	4.0

Table 9: Time-Course (Kinetic) Study Patients in Barbados: Patient 1016

Test Type	Days Post Onset				
	2	6	11	14	100
MAT Titer	0	400	3200	12800	3200
IgM ImmunoDOT positive dots	1.5	4.0	3.5	4.0	3.0
IHA Relative Titer	0.0	2.0	3.0	4.0	0.0

Table 10: Time-Course (Kinetic) Study Patients in Barbados: Patient 1259

Test Type	Days Post Onset				
	5	6	13	34	62
MAT Titer	6400	25600	51200	12800	
IgM ImmunoDOT positive dots	4.0	4.0	4.0	4.0	3.5
IHA Relative Titer	2.0	4.0	3.0	3.0	3.0

Table 11: Time-Course (Kinetic) Study Patients in Barbados: Patient 1386

Test Types	Days Post Onset			
	1	5	24	180
MAT Titer	3200	51200	51200	1600
IgM ImmunoDOT positive dots	4.0	4.0	3.5	1.5
IHA Relative Titer	1.0	3.0	3.0	0.0

CULTURE ISOLATION STUDIES

Leptospira organisms were cultured from the blood or urine of 12 patients in Hawaii and 24 patients in Barbados to confirm leptospirosis infections and to identify the infecting serovars. In Hawaii, 9 culture positive patients (75%) were IgM ImmunoDOT positive and 10 patients (80%) were MAT positive. In Barbados, all of 24 culture positive patients (100%) were IgM ImmunoDOT positive and 23 patients (95.8%) were MAT positive. Both tests demonstrated broad reactivity to infecting serovars as well as additional serovars identified by the MAT test but not isolated. The most common serovar isolated in the Hawaii study was australis and in the Barbados study the most common serovar isolated was bim. However, serovar expression may vary significantly over time and within a given region.

BIBLIOGRAPHY

1. **Levett, P N and Whittington, C U.** Evaluation of the indirect hemagglutination assay for the diagnosis of acute leptospirosis. *J Clin Microbiol.* 1998, Vol. 36, p. 11.

2. **Ribeiro, M A, Assis, C SN and Romero, E C.** Serodiagnosis of human leptospirosis employing ammunodominant antigen. *Serodiagn Immunother Infect Disease.* 1994, Vol. 6, p. 140.

3. **Turner, L H.** Leptospirosis II. *Trans Royal Soc Trop Med & Hygiene.* 1968, Vol. 62, p. 880.

4. **Pappas, M G, Ballou, R and Gray, M R.** Rapid serodiagnosis of leptospirosis using the IgM=specific dot ELISA: Comparison with the microscopic agglutination test. *Am J Trop Med Hyg.* 1985, Vol. 34, 2, p. 346.

5. **Case definitions for infectious conditions under Public Health Surveillance. *MMWR.* 1997, Vols. 46(No RR-10), p. 49.**

6. **Sulzer, C R, Glosser, J W and Rogers, F. Evaluation of an indirect hemagglutination test for the diagnosis of leptospirosis. *J Clin Microbiol.* 1975, Vol. 2, p. 218.**

7. **US Centers for Disease Control. *Manual Guide – Safety Management No. CDC–22 Decontamination of Laboratory Sink Drains to Remove Azide Salts.* Atlanta : Centers for Disease Control, 1976.**

8. **—. *HHS Publication No. (CDC) 93-8395, 3rd ed: Biosafety in Microbiological and Biomedical Laboratories.* Washington DC : US Government Printing Office, 1993.**

9. **US Department of Labor, Occupational Safety and Health Administration. *29 CFR Part 1910.1030, Occupational safety and health standards, bloodborne pathogens.***

10. **US Department of Health and Human Services. *HHS Publication No. (CDC) 21-11: Biosafety in Microbiological and Biomedical Laboratories. 5th ed.* Washington DC : US Government Printing Office, 2009.**

11. **World Health Organization. *Laboratory Biosafety Manual 3rd ed.* Geneva : World Health Organization, 1991.**

12. ***Epidemiology Symposium: Leptospirosis, a disease that is re-emerging on a worldwide basis.* Ashford, D. Houston : 49th Annual Meeting of American Society of Tropical Medicine and Hygiene, 2000.**

13. ***Assesssment of the clinical accuracy of laboratory tests using Receiver Operating Characteristics (ROC) plots.* s.l. : NCCLS, 1995. GP10-A.**

QUICK REFERENCE PROCEDURE

IMMUNODOT LEPTOSPIRA IGM

Set-Up
- Make sure Workstation is at temperature.
- Place reaction Vessels into slots in Workstation and add water to the water container.
- Place 2 mL Diluent (1) in Vessel #1; 2 mL Enhancer (2) in Vessel #2; 2 mL Conjugate (3) in Vessel #3; and 2 mL Developer (4) in Vessel #4.
- Wait 10 minutes

Procedure
- Add 10 µL serum to Vessel #1.
- Prewet assay strip in water for 30 - 60 seconds.
- Place strip in Vessel #1, mix, let stand 10 min.
- Remove strip, place in water, swish 5-10 sec.
- Place strip in Vessel #2, mix, let stand 5 min.
- Remove strip, place in water, swish 5-10 sec.
- Place strip in Vessel #3, mix, let stand 15 min.
- Remove strip, place in water, let stand 5 min.
- Place strip in Vessel #4, mix, let stand 5 min.
- Remove strip, place in water, swish, blot, dry, and read

To place an order for ImmunoDOT products, contact your local distributor, or call GenBio directly for the distributor nearest you and for additional product information.

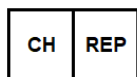For assistance, please call toll-free 800-288-4368.

GenBio

15222-A Avenue of Science
San Diego, CA 92128

EC	REP

MedEnvoy Global B.V.

Prinses Margrietplantsoen 33 - Suite 123

2595 AM The Hague

The Netherlands

CH	REP

MedEnvoy Switzerland
Gotthardstrasse 28
6302 Zug
Switzerland