A REVIEW OF LABORATORY TECHNIQUES FOR DETECTING LEPTOSPIROSIS

MONIKA JAIN, R. K. NIGAM, REENI MALIK*
Department of Pathology, Gandhi Medical College, Bhopal 462001 M. P. India
Email: drmonikajain18@gmail.com

ABSTRACT

Occupational and recreational exposure to leptospirosis occurs in both the developed and developing countries. Timely laboratory confirmation of the disease is necessary to ensure a favourable clinical outcome and it is not possible to diagnose leptospirosis with certainty, on clinical grounds alone due to its inconsistent manifestations. Dark-field microscopy has multiple limitations. Many serological tests are easy to perform with minimum skills and laboratory facilities. To make a reliable diagnosis, it is essential to use multiple techniques together or in succession. Microscopic agglutination test is considered the “gold standard” but requires paired sera, maintenance of live cultures of leptospires and is not useful for guiding early clinical management.

Though the Polymerase Chain Reaction requires special equipment and expertise, it is useful for diagnosing leptospirosis in the first week of illness. Culture provides definite proof of leptospiral infection and helps in identifying locally pathogenic serovars. However, it is not useful as a diagnostic tool because by the time diagnosis is made by culture, antibodies are already detectable by serological techniques.

Sero-surveillance helps in identifying high-risk individuals, high-risk geographical areas, outbreaks, animal reservoirs, new serological variants and their geographical distribution.

This review discusses various techniques for laboratory diagnosis of leptospirosis compared the technical limitations in terms of sensitivity, specificity and turnaround time. Based on the discussed facts, clinicians can decide the suitable technique of detections on case to case basis.

Keywords: ELISA, Leptospirosis, Microscopic agglutination test, PCR, Sero-diagnosis, Sero-surveillance.

INTRODUCTION

The estimated annual incidence of leptospirosis (per one million populations) for countries with tropical and temperate climates is 100-1000 and 1.0–1.0, respectively [1]. Inhabitants of developed countries, who travel to tropical countries or are occupationally and recreationally, exposed are also vulnerable to leptospirosis.

The clinical presentation ranges from non-specific febrile illness to severe icteric disease, acute renal failure, and adult respiratory distress syndrome. The symptoms and signs include high-grade fever, headache, retro-bulbar pain, transient maculopapular skin rashes, photophobia, sub-conjunctival suffusion, severe myalgia, prostration, oliguria, tender hepatomegaly and rarely, splenomegaly. It is not possible to diagnose the disease with certainty, on clinical grounds alone because of its protean manifestations [1]. If not diagnosed and treated at an early stage, the disease can result in fatal complications. Hence early laboratory confirmation of the disease is necessary [2].

Since the disease can also occur sporadically, health care providers need to have a high index of clinical suspicion so that the investigations are done at an early stage and all suspected cases are thoroughly investigated.

Dark-field microscopy

Leptospires are too thin to be visible under the light microscope, but are visible under dark-field microscopy and by silver impregnation techniques. In dark-field microscopy, leptospires are seen as silvery threads against a dark background. However, this method is technically demanding and requires skilled personnel to detect small numbers of leptospires. There is a high risk of false positive results because serum protein, fibrin strands and cellular debris in blood resemble leptospires. The concentration of leptospires in urine is too low to be detected by this method. Hence, dark-field microscopy is not recommended as a diagnostic tool and a positive result obtained by this method ought to be confirmed by other diagnostic tools [1]. Dark-field microscopy is used to examine leptospires in culture and to detect agglutination in the microscopic agglutination test (MAT).

Serological tests

After infection, seroconversion usually occurs in 10 days, but the duration may be variable [1]. IgM antibodies appear earlier than IgG antibodies and remain detectable at low titres for months or even years. Detection of IgG antibodies is even more variable. Genus-specific, serovar-specific and serogroup-specific antibodies produced by the host’s immune system react with leptospiral antigens, when patient’s blood is brought in contact with antigens in the serological test kits. Some tests use live leptospires as antigens, while others employ extracts of leptospires [1].

Several serological methods are used for the detection of IgM and IgG antibodies. Some are used as screening tests for leptospirosis. Each test has its own merits and limitations. Enzyme-linked Immunosorbent Assay (ELISA), Micro- Agglutination Test (MAT), Indirect Hemagglutination Test (IHA), Micro Capsule Agglutination Test (MCAT), and Lepto Dipstick are commercially available. These tests are easy to perform with minimum laboratory facilities. However, some of these tests require incubation for at least three hours [3, 4].

To make a reliable diagnosis, it is essential to use multiple techniques together or in succession. The antibody titre gradually increases during the course of illness, peaks and decreases after recovery. Weak serological reaction may be due to administration of high dose of antibiotics during early phase of illness, presence of non-specific antibodies, severe illness, immune suppression or very early or late phase of immune response.

An IgG titre of 1:100 can be present due to past infection and IgG antibodies may remain detectable for several months or years [5]. The results of serological tests should always be correlated with clinical presentation and history of risk factors [1].

Diagnosis of leptospirosis is confirmed by any one of the following criteria–

- Detection of leptospires in blood, cerebrospinal fluid, or urine,
- Suggestive clinical symptoms associated with either--[a] four-fold increase in initial titres by Micro-Agglutination Test (MAT) or a
single MAT titre ≥ 400, or [b] detection of specific IgM antibodies by Dot ELISA or Dipstick ELISA [6, 7].

Macroscopic slide agglutination test (MSAT)

This test is rapid and reliable for screening purposes. A fixed quantity of concentrated killed antigen is mixed with a fixed quantity of patient’s serum sample on a slide. Presence of agglutination is observed with the naked eye. Agglutination (formation of clumps) indicates presence of genus-specific leptospira antibodies in the serum sample (i.e. positive test). It is a good screening test with high sensitivity, but has low specificity. MSAT is easy to perform and read. The antigen is stable for six months at 4 ° Celsius. In the early stage of the disease, it is more sensitive than MAT. When the antigen is coloured with a drop of Gentian violet, the visual reading of the result is improved. The frequency of false negative results (due to auto agglutination of antigen when old cultures are used) is low [8, 9].

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a rapid, sensitive, and specific test and can be designed to detect IgM antibodies. Several techniques of ELISA are available, depending on the type of antigens and reagents used. Conventional ELISA techniques involve the use of antigen-coated micro wells and a sonicated preparation of different antigens. The type of antigens used depends on the manufacturer of the test kit. Though in-house ELISA is a cheap alternative to commercially available ELISA kits, it requires standardization. The conventional tests have evolved to more convenient and rapid ELISA-based tests for detecting leptospira-specific IgM antibodies (dot ELISA and dipstick ELISA), and for detecting leptospira antigens (sandwich ELISA).

Dot ELISA

Minute quantities of the antigen are dotted on nitrocellulose discs and the sera are reacted with chromogenic substrate that can be precipitated. The test is rapid (about two hours), economizes on quantity of antigen used, and can be performed in field settings due to its portability [10].

Dipstick ELISA

The Dipstick assay is easy to perform quickly and it does not require electricity or special equipment. An additional advantage is that the dipstick and the staining reagent can be stored for prolonged periods, the test cannot differentiate between agglutinated leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.

MAT may indicate the sero-group to which the infective serovar belongs, but only rarely identifies it. Both IgM and IgG antibodies are detected but since high titres of IgG antibodies may persist for prolonged periods, the test cannot differentiate between agglutinating antibodies due to current, recent or past infection [15].

Leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.

Leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.

Leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.

Leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.

Leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.

Leptospira strains for use as antigens. It is never possible to ensure that the panel of cultures is complete since new, unidentified strains may cause illness. MAT cannot be standardized (unlike ELISA) since live leptospira are used as antigens and several factors (age and density of antigen cultures) affect the agglutination titres. There may be day-to-day variations in test results. To overcome this problem, paired samples should be tested together.
concordant with that of IgM ELISA [17]. Its sensitivity and specificity at different stages of illness are compared in the table.

Disadvantages

This test cannot detect the infecting serovar. The possibility of weak staining of the antigen band is overcome by repeating the test with the same sample (or, if possible, with a fresh sample) [3]. The test is expensive if required for large-scale use. The test requires incubation for three hours, before the results can be read [3, 11].

Lepto dri dot

Lepto Dri Dot, a card agglutination test (Royal Tropical Institute, Amsterdam, The Netherlands) for the rapid diagnosis of leptospirosis, is based on the binding of leptospira-specific antibodies in patients’ serum to the broadly reactive antigen coated on latex particles, leading to agglutination [18]. The individually wrapped agglutination cards contain a stable, dried detection agent. The method involves simply suspending the dried reagent with a drop of serum. The result is obtained within 30 seconds [19]. Its sensitivity and specificity are compared in the table.

Advantages

Relatively low skilled personnel can perform the test without sophisticated equipment. Unlike ELISA, it need not be done in batches. Test with a single sample gives reasonably reliable information by the fifth day of illness. Lepto Dri Dot is commercially available [18, 19]. The test kit and reagents have long shelf life even at tropical temperatures. Hence, this test is suitable for use as a rapid screening test in field settings [18].

Lepto lateral flow

Lepto Lateral Flow (Royal Tropical Institute, Amsterdam, The Netherlands) is a rapid diagnostic test based on the binding of leptospira-specific IgM antibodies to the broadly reactive heat-extracted antigen prepared from non-pathogenic Patoc 1 strain. These bound antibodies are detected with an anti-human IgM gold conjugate contained within the test devices [20].

This test uses stabilized components and is performed by adding serum and sample fluid to the sample well of the assay device. The assay is read after ten minutes and staining of the test line indicates positive result [20]. The test can also be performed at the bedside of the patient, using a drop of whole blood obtained by finger prick. The test kit and sample solution do not require any special storage. Hence this test is suitable for use in peripheral health centres and in field settings [16, 21].

Smits et al. [20] reported that sensitivity of the assay varied with the stage of the disease. The sensitivity and specificity was found to be 85.8% and 93.6%, respectively and found 91.9% agreement with those of IgM ELISA [19]. Sehgal et al. [16] have reported that the sensitivity and specificity for Lepto lateral flow were comparable to that for IgM ELISA and Lepto dipstick tests (table 1).

<table>
<thead>
<tr>
<th>Diagnostic technique</th>
<th>Stage of illness</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepto dipstick</td>
<td>First ten days</td>
<td>84.5%</td>
<td>97.5%</td>
</tr>
<tr>
<td></td>
<td>10-30 days</td>
<td>92.1%</td>
<td>94.4%</td>
</tr>
<tr>
<td></td>
<td>First week</td>
<td>48.6%</td>
<td>85.1%</td>
</tr>
<tr>
<td></td>
<td>2-4th week</td>
<td>87.7%</td>
<td>85.1%</td>
</tr>
<tr>
<td></td>
<td>4-8th week</td>
<td>90%</td>
<td>90.6%</td>
</tr>
<tr>
<td>Lepto dri dot</td>
<td>First week</td>
<td>67.6%</td>
<td>66.0%</td>
</tr>
<tr>
<td></td>
<td>2-4th week</td>
<td>85.5%</td>
<td>80.0%</td>
</tr>
<tr>
<td></td>
<td>First ten days</td>
<td>72.3%</td>
<td>93.9%</td>
</tr>
<tr>
<td></td>
<td>Later stages</td>
<td>88.2%</td>
<td>89.8%</td>
</tr>
<tr>
<td>Lepto lateral flow</td>
<td>First week</td>
<td>52.9%</td>
<td>93.6%</td>
</tr>
<tr>
<td></td>
<td>2-4th week</td>
<td>86.0%</td>
<td>89.4%</td>
</tr>
<tr>
<td>IgM ELISA</td>
<td>First week</td>
<td>50.0%</td>
<td>78.7%</td>
</tr>
<tr>
<td></td>
<td>2-4th week</td>
<td>87.7%</td>
<td>87.2%</td>
</tr>
<tr>
<td>Indirect hemagglutination</td>
<td>---</td>
<td>100%</td>
<td>94%</td>
</tr>
<tr>
<td>assay</td>
<td>---</td>
<td>92.2%</td>
<td>94.4%</td>
</tr>
</tbody>
</table>

Indirect Hemagglutination assay (IHA)

The Indirect Hemagglutination Assay (IHA) uses a soluble antigen from serotype Patoc to sensitize sheep erythrocytes, which are then fixed with glutaraldehyde. The assay is easy to perform, and does not require specialized equipment or highly skilled personnel. The sensitized fixed erythrocytes may be stored for at least one year [22]. The sensitivity and specificity IHA, as reported by two studies [23, 24] are outlined in the Table. The sensitivity of IHA was found to be substantially lower in a study from Hawaii, as compared to that reported in previous studies, particularly in the early phase of illness [25].

Micro capsule agglutination test (MCAT)

Micro Capsule Agglutination Test (MCAT) employs chemically stable microcapsules instead of sheep erythrocytes. Sonically disrupted antigens of leptospira are sensitized to microcapsules treated with glutaraldehyde. The sensitized microcapsule antigens are stable for at least one year. When coupled with mixed antigens, the microcapsules can be used as a screening test for infections caused by several serovars of leptospira. The test is simple to perform, easy to read and does not require any special training or equipment [26, 27].

As compared to MAT, the sensitivity and specificity of the test were 84.7% and 87.0%, respectively. During the early stages of the disease, the test had a higher sensitivity than MAT (75% vs 58.3%), though its specificity was less than that of MAT (83.3% vs 100%). The sensitivity declined to 61% 3-4 weeks after the onset of illness. MCAT is useful for early diagnosis of leptospirosis [26]. In some cases, the test may yield a positive result earlier in the course of the disease, as compared to MAT or IgM ELISA. However, MCAT may not detect antibodies against some serovars in certain regions [28].

Other serological tests for Leptospirosis

- In the Complement fixation test (CFT) standardization of reagents is a technically complex procedure. Its other limitations include short shelf life of reagents, and anti-complement activity of sera.
- Indirect fluorescent antibody test (IFAT) requires fluorescent microscope (expensive) and is not used in routine diagnostic laboratories.
- Counter Immuno electrophoresis (CIEP) is not commercially available.

Choice of serological test

The available serological tests are genus-specific and serogroup/serovar-specific. Genus-specific tests are more sensitive, less specific, and rapid. The sero-group/serovar-specific tests are useful.
for confirming diagnosis and also for sero-epidemiological purposes (identifying the sources of infection, reservoirs, and the circulating serovars in the community).

Ideally, the serum sample is to be screened using a rapid or simple test that detects IgM antibodies. Subsequently, diagnosis is to be confirmed by the “gold standard” i.e. microscopic agglutination test (MAT), which is not available in most clinical laboratory settings.

**Limitations of serological tests**

The presence of IgG antibodies does not indicate recent infection, while IgM antibodies indicate acute infection. Positive serological tests, per se, do not indicate current infection since some antibodies persist for a long time. If only one serum sample is sent to the laboratory, several fold rise above the cutoff point indicates current or recent infection. However, IgG antibodies may remain detectable for several months or years. The results of serological tests should always be correlated with clinical presentation and history of risk factors. The only definitive proof of leptospiral infection is the isolation of pathogenic leptospires [1].

**Polymerase chain reaction (PCR)**

PCR is a rapid method that amplifies specific segments of leptospiral DNA (in clinical and post-mortem samples) to detectable levels [1]. It useful during the diagnostic “window” in the first week of illness, when the antibodies cannot be detected by the other methods [13]. Primers (short DNA sequences specific for leptospires) are combined with heat-stable DNA polymerase in the presence of nucleotides and subjected to temperature cycles. This amplifies a stretch of leptospiral DNA, which is easily detected in gels. Labelled probes are also highly specific tools for detection. Many PCR techniques are available. In a culture-confirmed case, the leptospiral DNA persists in serum for an average duration of 12 days, and for a maximum of 6 days [29].

The assay can detect as few as ten organisms in variety clinical samples like urine and cerebrospinal fluid [30]. This test can also detect leptospires in aqeous humour during the ocular complications of the disease [29]. For the early diagnosis of leptospirosis, PCR analysis using urine samples can be more successful than that using serum. This technique may also be used for detecting long-term urinary shedding of leptospires by patients who have been treated for the disease [31].

PCR is an efficient tool for rapid diagnosis during the first ten days of the disease, especially when the clinical manifestations are confusing in early phase of infection [29, 32, 33]. However, the assay requires special equipment and skilled personnel. False positive results may be obtained even if a minute quantity of extraneous DNA contaminates the sample [1]. Leptospira detection using PCR could improve the management of patients presenting to hospital within the first few days of the onset of symptoms of leptospirosis, although cost represents a barrier to its implementation in resource-restricted countries [34]. However, real-time PCR method for the detection of the gene encoding the surface lipoprotein Lipl32 found to be a reliable, sensitive, and rapid method for the detection of the acute form of leptospirosis. [35]. Also, The sequence polymorphism of diagnostic PCR products proved useful in presumptively identifying the infecting Leptospira strains [36]. Recently, TaqMan®-based multi-gene targeted real-time PCR approach yielding high sensitivity and specificity for the direct detection and differentiation of the most relevant pathogenic Leptospira species in animal samples has been developed, suitable for introduction into the routine diagnostics of veterinary laboratories [37].

**Diagnosis by culture**

Leptospires are fastidious organisms with complex growth requirements. Since vitamin B1, B2, and long-chain (containing more than 15 carbon atoms) fatty acids are the only known essential nutrients for leptospires, rabbit serum that contains the highest concentration of bound vitamin B12 is used in culture media. Leptospires use fatty acids (not carbohydrates) as a source of energy, but cannot synthesize fatty acids. Pyruvate, a non-essential nutrient, enhances growth of fastidious leptospires. In contrast to most other bacteria, leptospires do not use external sources of pyrimidine bases for incorporation into their DNA or RNA. Being resistant to antimicrobial action of 5-fluorouracil (a pyrimidine analogue), this drug is used in selective media to isolate leptospires from contaminated clinical samples [1].

**Advantages**

Culture provides definite proof of leptospiral infection. Isolated leptospires can be sero-typed to identify locally pathogenic serovars and to detect new serovars (useful as tool for surveillance in public health). Culture is a useful for post mortem diagnosis of infection in patients who died in early phase of infection, before antibodies could be detected [1].

**Disadvantages**

Leptospires grow slowly with a maximum doubling time of 6-8 hours. Optimal temperature (28-30 °Celsius) has to be maintained. By the time diagnosis is made, antibodies are already detectable by serological techniques. Thus, it is not useful as a diagnostic tool for treating patients [1].

**Sero-surveillance**

Microscopic Slide Agglutination Test (MSAT) and Indirect Hemagglutination Assay (IHA) are screening tests for serological surveillance of leptospirosis [11]. Since Lepto Dipstick and IgM ELISA have higher positive predictive value (PPV) during all stages of illness, these are also useful as screening tests [3]. For these screening tests, samples of capillary blood are aseptically collected by finger prick on filter paper and are allowed to dry at room temperature.

In countries that have inadequate notification systems, sero-surveillance can be useful in identifying high-risk individuals, high-risk geographical areas, outbreaks, animal reservoirs, new serological variants and their geographical distribution, and new strategies for prevention and control of the disease.

For sero-surveillance in humans, the sources of serum samples are hospital patients with clinical manifestations resembling that of leptospirosis, known risk groups and random samples of blood. Periodic re-examination of the same risk group in a population may provide information on sero-conversion. The participants in such a survey may be asked about history of exposure to possible risk factors during the time interval between consecutive examinations [1].

Serological testing of animals is useful in determining primary reservoirs in a locality, though animals might harbour leptospires without having detectable antibodies in serum. Since leptospires are not evenly distributed in an environment, negative culture results do not exclude the presence of pathogenic leptospires. The environment or surface water may have been free of pathogenic leptospires at the time of sample collection, but may subsequently get contaminated by urine of infected animals [1].

**CONFLICT OF INTERESTS**

Declared None

**REFERENCES**