Academic Sciences

International Journal of Current Pharmaceutical Research

ISSN- 0975-7066 Vol 7, Issue 3, 2015

Review Article

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

Received: 16 Mar 2015, Revised and Accepted: 05 Apr 2015

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 1.0–10.0, 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 Assay (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 at tropical temperatures [11, 12]. ELISA is used as a screening test for leptospirosis [3]. Generally, an antigen derived from serovar hard jo of *Leptospira interrogans* is used with horseradish peroxidase. An anti-species antibody conjugated to an enzyme is added. The activity of the enzyme is determined by adding a specific chromogenic substrate. Within a certain range of concentration, intensity of colour reaction is proportional to quantity of antibody present in the serum sample. IgM titre of 1:80 to 1:100 is considered suggestive of leptospiral infection and the diagnosis is confirmed by MAT.

Sandwich ELISA

Two-tip nitrocellulose dipstick (after loading with conjugate and incubating at room temperature for 45 minutes) is incubated in substrate solution for 3-5 minutes till a colored dot appears at the upper tip of the dipstick. Development of colored dots in both upper and lower tips of the dipstick indicates a positive result. The test is negative if only the upper tip of the dipstick shows a colored dot.

Advantages of ELISA

ELISA can detect IgM antibodies about 6-8 days after onset of first clinical manifestations. This may be delayed if antibiotic treatment has been started. Since the titre of IgM antibodies rises and falls rapidly, a positive ELISA test is suggestive of current infection. Since a single genus-specific antigen is used, ELISA can be standardized, unlike MAT. There is no need to maintain panel of cultures in local laboratory since ELISA kits are commercially available [1].

Disadvantages of ELISA

Being less specific than MAT, it is used as screening test. In co-infection with other pathogens, weak cross-reactions may occur. Since it is based on a genus-specific antigen, ELISA does not indicate the infecting serovar [WHO-ILS]. It requires special expertise and equipment. Continuous electric supply is needed for refrigerating reagents and for performing the test [3].

Microscopic agglutination test (MAT)

Only experienced laboratories that can maintain a large number of live, locally prevalent strains of leptospira use the MAT, which employs multiple live antigens [13]. Ideally, two consecutive serum samples are to be examined for sero-conversion. Alternatively a four-fold (or greater) rise in titre is considered significant. If a single serum sample is sent to the laboratory, different cut-off points varying from 1:100 to 1: 800 are applied in different localities [1].

Several serial dilutions of serum are mixed with leptospires (antigens). If anti-leptospiral antibodies (both IgM and IgG) are present in the serum, the leptospires tend to agglutinate. Movement of the free ends of agglutinated leptospires is visible by dark-field microscopy.

Advantages

Currently, this MAT is considered the "gold standard" for detecting leptospiral antibodies because of its high diagnostic specificity in comparison with currently available serological tests [1, 6]. MAT test can be standardized in a diagnostic laboratory and used in conjunction with an IgM ELISA [14].

Disadvantages

MAT is technically demanding, time consuming, and requires a well-equipped laboratory and maintenance of large number of live 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 leptospiras 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].

MAT titres are usually low in the first few weeks of illness, making diagnosis based on a single sample difficult. A single MAT has been found to have a lower sensitivity, as compared to that for lepto dipstick and IgM ELISA. Thus, MAT does not appear to be the serological test of choice for routine use. A genus-specific screening test like ELISA is used before performing MAT.

Lepto dipstick

This assay detects leptospira-specific IgM antibodies in human sera. The dipstick contains two horizontal bands–[a] lower band does the antigen band comprise broadly reactive leptospira, and [b] upper band is the internal control bound to a nitrocellulose strip. An anti-human IgM-dye conjugate detects human leptospira-specific IgM antibodies that bind to the leptospira antigen (lower band).

Dipping white end of the test strip in the dipstick fluid for one minute moistens the dipstick. The white end of the dipstick is then incubated for 3 hours at room temperature in a mixture containing detection agent and serum. Development of a visible reddish coloured antigen band indicates a positive test. If the test is negative, the reddish coloured antigen band does not develop. The upper band (internal control) should stain in all cases. If it does not, the assay is invalid.

Advantages

Lepto dipstick requires only a single dilution, is easy to perform and read and does not require any special equipment. The dipsticks and reagents have a long shelf life, even at room temperature. Hence, it can be used as a screening test in field settings [16].

In an International multi-centre evaluation, the dipstick assay detected a broad variety of serogroups and the results were

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 assay can be also 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 1: Comparision of sensitivity & specificity obtained with

Diagnostic technique	Stage of illness	Sensitivity	Specificity
Lepto dipstick	First ten days	84.5%	87.5%
	10-30 days	92.1%	94.4%
	First week	48.6%	85.1%
	2nd-4th week	87.7%	85.1%
	----	98%	90.6%
Lepto dri dot	First week	67.6%	66.0%
	2nd-4th week	85.5%	80.0%
	First ten days	72.3%	93.9%
	Later stages	88.2%	89.8%
Lepto lateral flow	First week	52.9%	93.6%
	2nd-4th week	86.0%	89.4%
IgM ELISA	First week	50.0%	78.7%
	2nd-4th week	87.7%	87.2%
Indirect hemagg-lutination assay	----	100%	94%
	----	92.2%	94.4%

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 sero-group/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 56 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 aqueous 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 B_1, B_{12} 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 B_{12} 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

1. World health organization and international leptospirosis society. Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control. Geneva: World Health Organization; 2003. p. 9-16, 63-80.
2. Sehgal SC, Murhekar MV, Sugunan AP. Outbreak of leptospirosis with pulmonary involvement in North Andaman. Indian J Med Res 1995;102:9-12.
3. Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LEPTO Dipstick: a rapid and simple method for sero-diagnosis of acute leptospirosis. Trans R Soc Trop Med Hyg 1999;93:161-4.
4. Vijayachari P, Sugunan AP, Sehgal SC. Evaluation of lepto dri dot as a rapid test for the diagnosis of leptospirosis. Epidemiol Infect 2002;129:617-21.
5. Mitra DK. Leptospirosis. In: Sainani GS ed. API Textbook of Medicine. 6th edn. Mumbai: Association of Physicians of India; 1999. p. 62.

6. Gulati S, Menon S, Kabra M, Chaudhary R, Kalra V. Leptospirosis–A case report. Pediatrics Today 2002;5(7):428-33.
7. Andre VL, Diamen D, Jaime RT. Leptospirosis in Latin America. Infect Dis Clin North Am 2000;14:23-39.
8. Galton MM, Powers DK, Hall D, Cornell RG. A rapid macroscopic screening test for the serodiagnosis of leptospirosis. Am J Vet Res 1958;19:505-12.
9. Wolff JW, Bohlander HJ. Screening tests in human serum samples with *Leptospira biflexa* antigens incorporated in Galton's macroscopic slide test. Trop Geogr Med 1967;19:63-9.
10. Watt G, Alquiza LM, Padre LP, Tuazon MI, Laughlin LW. The rapid diagnosis of leptospirosis: a prospective comparison of the dot enzyme-linked immunosorbent assay and the genus-specific microscopic agglutination test at different stages of illness. J Infect Dis 1988;157(4):840-42.
11. Gussenhoven GC, van der Hoorn MAWG, Goris MGA, Terpstra WJ, Hartskeevi RA, Mol BW, *et al*. LEPTO Dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin m antibodies in Human sera. J Clin Microbiol 1997;35(1):92-7.
12. Smits HL, Hartskeerl RA, Terpstra WJ. International multi-centre evaluation of dipstick assay for human leptospirosis. Trop Med Int Hlth 2000;5(2):124-8.
13. Adler B, Faine S. The antibodies involved in human immune response to leptospiral infection. J Med Microbiol 1978;11:387-400.
14. Shekatkar SB, Harish BN, Menezes GA, Parija SC. Clinical and serological evaluation of Leptospirosis in Puducherry, India. J Infection Developing Countries 2010;4(3):139-43.
15. Sehgal SC. Emergence of Leptospirosis as a public health problem. Proceedings of the Round table Conference on Leptospirosis. Pune 1998;3:7-17.
16. Sehgal SC, Vijayachari P, Sugunan AP, Umapathi T. Field application of Lepto Lateral Flow for rapid diagnosis of leptospirosis. J Med Microbiol 2003;52:897-901.
17. Smits HL, Ananyina YV, Chereshky A, Dancel L, Lai-A-Fat RFM, Chee HD, *et al.* International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. J Clin Microbiol 1999;37(9):2604-9.
18. Smits HL, van der Hoorn MAWG, Goris MGA, Gussenhovoen GC, Yersin C, Sasaki DM, *et al.* Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. J Clin Microbiol 2000;38(3):1272-5.
19. Smits HL, Chee HD, Eapen CK, Kuriakose M, Sugathan S, Gasem MH, *et al.* Latex based rapid and easy assay for human leptospirosis in a single test format. Trop Med Int Health 2001;6(2):114-5.
20. Smits HL, Eapen CK, Sugathan S, Kuriakose M, Gasem MH, Yersin C, *et al.* Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin Diagn Lab Immunol 2001;8(1):166-9.
21. Eapen CK, Sugathan S, Kuriakose M, Abdoel T, Smits HL. Evaluation of the clinical utility of a rapid blood test for human leptospirosis. Diagn Microbiol Infect Dis 2002;42(4):221-5.
22. Sulzer CR, Jones WL. Evaluation of a hemagglutination test for human leptospirosis. Appl Microbiol 1973;26(5):655-7.
23. Levett PN, Whittington CU. Evaluation of the Indirect Hemagglutination Assay for the diagnosis of acute leptospirosis. J Clin Microbiol 1998;36(1):11-4.
24. Levett PN, Branch SL, Whittington CU, Edwards CN, Paxton H. Two methods for rapid serological diagnosis of acute leptospirosis. Clin Diagn Lab Immunol 2001;8(2):349-51.
25. Effler PV, Domen HY, Bragg SL, Aye T, Sasaki DM. Evaluation of the Indirect Hemagglutination Assay for the diagnosis of acute leptospirosis in Hawaii. J Clin Microbiol 2000;38(3):1081-4.
26. Arimitsu Y, Kobayashi S, Akama K, Matuhasi T. Development of a simple serological method for diagnosing leptospirosis: a micro capsule agglutination test. J Clin Microbiol 1982;15(5):835-41.
27. Sehgal SC, Vijayachari P, Subramaniam V. Evaluation of leptospiral micro capsule agglutination test (MCAT) for serodiagnosis of leptospirosis. Indian J Med Res 1997;106:504-7.
28. Arimitsu Y, Kmety E, Ananyina Y, Baranton G, Ferguson IR, Smythe L, *et al.* Evaluation of the one-point micro capsule agglutination test for diagnosis of leptospirosis. Bull WHO 1994;72(3):395-9.
29. Mérien F, Baranton G, Perolat P. Comparison of polymerase chain reaction with micro agglutination test and cluture for diagnosis of leptospirosis. J Infect Dis 1995;172(1):281-5.
30. Mérien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for detection of Leptospira spp in clinical samples. J Clin Microbiol 1992;30(9):2219-24.
31. Bal AE, Gravekamp C, Hartskeerl RA, de Meza-Brewster J, Korver H, Terpstra WJ. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol 1994;32(8):1894-8.
32. Gravekamp C, Van de Kemp H, Franzen M, Carrington D, Schoone GJ, Van Eys GJ. *et al.* Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiol 1993;139(8):1691-700.
33. Brown PD, Gravekamp C, Carrington DG, Van de Kemp H, Haartskeerl RA, Edwards CN, *et al.* Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. J Med Microbiol 1995;43(2):110-4.
34. Thaipadungpanit J, Chierakul W, Wuthiekanun V, Limmathurotsakul D, Amornchai P, Boonslip S, *et al.* Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipL32 genes for human leptospirosis in Thailand: a case-control study. PLoS One 2011;6(1):e16236.
35. Cermakova Z1, Kucerova P, Pliskova L, Kubickova P. Real-time PCR method for the detection of the gene encoding surface lipoprotein LipL32 of pathogenic Leptospira: use in the laboratory diagnosis of the acute form of leptospirosis. Scand J Infect Dis 2013;45(8):593-9.
36. Perez J, Goarant C. Rapid Leptospira identification by direct sequencing of the diagnostic PCR products in New Caledonia. BMC Microbiol 2010;10:325.
37. Ferreira AS, Costa P, Rocha T, Amaro A, Vieira ML, Ahmed A, *et al.* Direct detection and differentiation of pathogenic Leptospira species using a multi-gene targeted real time PCR approach. PLoS One 2014;9(11):e112312.