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Clinical Laboratory Diagnosis of Human Leptospirosis

Pejvak Khaki[1,*]

[1]Reference Laboratory for Leptospira, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, IR Iran

*Corresponding author: Pejvak Khaki, Reference Laboratory for Leptospira, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, IR Iran. Tel: +98-2634502849, Fax: +98-2634452194, E-mail: P.khaki@rvsri.ac.ir

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Abstract

Context: Leptospirosis is a worldwide zoonotic infection which appears to be a re-emerging health problem. The clinical features of the disease are broad ranging, but are often similar to those of other infections. As a result, the accuracy of a clinical diagnosis of leptospirosis is low and confirmation requires the use of laboratory tests.

Evidence Acquisition: The disease is usually diagnosed in the laboratory by different methods such as direct microscopy, culture, serological methods and molecular methods. The microscopic agglutination test (MAT) is considered the reference test among the several serological methods for leptospirosis diagnosis. However, isolation and identification of the microorganism allows for definitive diagnosis, and provides for epidemiological and prophylactic studies of this disease. Therefore, culture is a golden standard method. Polymerase chain reaction is a rapid, sensitive and specific means of detecting leptospiral infection, in contrast to serology tests. Further benefit is the ability to identify early infection especially during the first few days of the disease even before antibodies are detectable.

Conclusions: Choice of test for diagnosis of leptospirosis depends on the stage of the disease. An ideal test will need to discriminate between leptospirosis and a broad spectrum of diseases that cause acute febrile illness and have overlapping clinical presentations. Although detection of antibodies is by itself no proof of a current infection, serological methods (such as MAT and ELISA) are often the most appropriate diagnostic methods.

Keywords: Leptospirosis, Laboratory Diagnosis, MAT, ELISA, PCR

1. Context

Leptospirosis is a zoonosis infectious disease caused by the pathogenic leptospires that are transmitted directly or indirectly from animals to humans (1).

The disease has been recognized as an important re-emerging public health problem with worldwide distribution but is more common in tropical and subtropical regions with high rainfall and flooding (2, 3). The disease is found mainly wherever humans come into contact with the urine of infected animals or a urine-polluted environment. Leptospirosis is an occupational disease which occurs in both industrialized and developing countries (1, 4).

In Iran, leptospirosis was first reported by Rafyi and Maghami in 1959 (5). The prevalence of leptospirosis in the northern provinces of Iran which are humid with heavy rainfall is high (6, 7).

Leptospira gain entry into the blood stream via cuts, skin abrasions or mucus membranes (8). Leptospirosis have evolved ways to escape the immune defense, pathogenic leptospires are able to translocate through cell monolayers at a rate significantly greater than that of nonpathogenic leptospires (9). The rapid translocation of pathogenic leptospires between mammalian cells allows the bacteria to quickly reach the bloodstream and disseminate to different organs, virulent leptospires can rapidly enter kidney fibroblast and induce a programmed cell death (10). The death in acute leptospirosis occurs due to multiple organ failure (kidney, liver, lung lesions) (11).

The disease may present with a wide variety of clinical manifestations. These may range from a mild "flu"-like illness to a serious and sometimes fatal icteric (Weil's syndrome) disease. It may also mimic many other febrile diseases, e.g. dengue fever and other viral hemorrhagic diseases (1, 12-14). Icterus (jaundice) is a relatively common symptom in leptospirosis but is also found in many other diseases involving the liver such as the various forms of hepatitis (9). The diagnosis is confirmed by laboratory tests, but these are not always available, especially in developing countries like Iran. For these reasons, leptospirosis is overlooked and underreported in many areas of the world (15, 16).

Therefore, a rapid and early diagnosis is critical with regard to initiation of appropriate treatment and control of the disease in human (17). Laboratory support helps to confirm leptospirosis and is necessary for epidemiological study in community (18).

The disease is usually diagnosed in the laboratory by detecting antibodies (serodiagnosis), culturing the bacteria

from blood, urine or tissues, demonstrating the presence of leptospires in urine and tissues or other methods may be available in some centers, e.g. the polymerase chain reaction (PCR) and immune-staining (19).

Recently different diagnosis methods have been developed for detection of leptospirosis in human. This review briefly explained the diagnostic of leptospirosis in human with a strong emphasize on the recent advances in diagnostic method.

2. Evidence Acquisition

2.1. Direct Microscopy

Leptospires are thin, coiled and motile in fluids such as blood or urine using dark-field microscopy (2). Leptospires can be concentrated in blood or urine by differential centrifugation. Although this technique is described in textbooks as a useful method of demonstrating leptospires in fluids, it has sometimes proved to be of doubtful value even in the hands of very experienced staff (8).

Direct microscopy of blood is not recommended as a routine method because artifacts such as serum protein, fibrin strands and cell debris in blood make mistake with leptospires (18). This technique is particularly useful for observing leptospires in culture, particularly when they are present in large numbers, and for observing agglutination in microscopic agglutination test (MAT) (18, 20, 21).

Phase contrast microscopy is also useful for visualizing leptospires in the laboratory, but, because of its technical limitations in thick suspensions and its optical characteristics, it has no practical purpose whenever dark-field microscopy is available (15, 18).

Leptospires cannot be stained, or only weakly, by Gram-staining. Silver staining may also give satisfactory results. Immunostaining methods, such as direct immunofluorescence, and immunoperoxidase staining, for diagnosis of lepropires have been used. Staining methods can be useful for in postmortem diagnosis on either “fixed” or “unfixed” tissues. However, all staining methods suffer from the same shortcomings as dark-field microscopy, i.e. a high risk of false-positive and false-negative diagnoses. Therefore, the results of dark-field microscopy of clinical material should always be confirmed by other tests (19, 21).

2.2. Culture

Diagnosis of leptospirosis may be accomplished by direct detection of the organism or its components in body fluid or tissues, by isolation of leptospires in cultures, *Leptospira* is fastidious and its isolation from clinical samples and culturing the organism is laborious and requires both special media and incubate for several weeks (22).

Different media have been used for the cultivation of leptospires but a commonly used medium is Ellinghausen- McCullough-Johnson-Harris (EMJH) medium, which contains 1% bovine serum albumin, 10% *Leptospira* enrichment (or 8 - 10% rabbit serum) and tween 80 (source of long-chain fatty acids), which are available commercially (14, 23, 24).

Media are made selective by the addition of several antibiotics such as 5-fluorouracil and neomycin sulphate, polymyxin B, nalidixic acid, rifampicin, vancomycin and amphotericin B. However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospires, and some strains of leptospires will not grow in selective media containing multiple antibiotics (25).

The isolation of leptospires depends on the choice of the samples and the stage of the disease. In leptospiraemic phase, the most suitable sample is blood. Blood should be cultured in the first 10 days of the illness and before antibiotics are given (26).

During the leptospiruria phase characterized by increasing concentrations of antibodies (after about 1 week from onset) the urine and the kidney (renal cortex) postmortem are the most suitable samples for the isolation of leptospires. Since urine is acid and decreases the viability of leptospires, it should be inoculated into medium within 2 hours after voiding and urine samples should be neutralized with sodium bicarbonate or using phosphate-buffered bovine serum albumin (BSA) solution (19, 27).

Leptospires may be isolated cerebrospinal fluid during the first weeks of illness. In fatal cases of human and animal leptospirosis, the organisms may be cultured from different postmortem tissues (liver or kidney). Leptospires may also be successfully isolated from aborted animal fetuses (27).

Cultures should be incubated between 28°C and 30°C for several weeks, because initial growth may be very slow (14, 23). The time required for detection of a positive culture varies with the leptospiral serovar and the number of organisms present in the sample. Less fastidious serovars (e.g. Pomona and Grippotyphosa) may result in positive cultures as soon as 7 - 10 days after inoculation; other serovars (e.g. Hardjo and Bratislava) may take much longer (4 - 6 months). Unfortunately, leptospires grow slowly so that, culture does not suitable for rapid diagnosis in the early phase of the disease. It is also a relatively insensitive diagnostic method. However, isolation of pathogenic leptospires is proof of an infection and culture is golden standard method (28, 29). Also, isolated leptospires can be typed to identify serovars and it is useful in the epidemiological studies of local pathogenic serovars (30).

2.3. Serological Methods

Current methods for the direct detection of leptospires are either slow or of limited reliability so that serology is often the most appropriate diagnostic method. Moreover, in practice, patients often seek medical care or are

admitted in hospitals when they have already been ill for a sufficiently long time to have produced detectable antibodies (31). However, detection of antibodies is by itself no proof of a current infection as some antibodies may persist for long periods after an infection. Generally, seroconversion (first sample, no detectable titre, the second sample, positive, i.e. above the cut-off point) or a four-fold or higher rise in titre in successive serum samples is considered to be diagnostic proof of recent infection (32). Serological information are important in the diagnostic process but must always be considered in conjunction with the clinical presentations and epidemiological data (a history of possible exposure and presence of risk factors) (33).

Recently two tests are used for the serological diagnosis of leptospirosis which includes MAT and ELISA (34).

2.3.1. Microscopic Agglutination Test

The microscopic agglutination test (MAT) is a simple micro-agglutination method, which determines agglutinating antibodies in the serum of a patient by mixing it in various dilutions with live different leptospiral antigens. The agglutination is observed using dark-field microscopy (20). *Leptospira* may be pathogenic or nonpathogenic, and over 250 different serovars of pathogenic leptospires are known. These are assigned to serogroups on the basis of their antigenic homology. Serovars from each serogroup are pooled and used as antigens for the MAT so that each serum is reacted with as many different leptospiral serovars as possible (35).

Patients usually produce agglutinating antibodies (IgM and IgG classes) against the infecting serovar. IgM antibody can be detected on days 6 to 10 after disease onset, peaks within 3 - 4 weeks and remains detectable for months to years (34). In early phase of the illness antibodies that cross-react with other serovars is also often observed which decrease relatively quickly, after months, while serogroup- and serovar-specific antibodies may persist for a much longer time, often for years (20).

The standard criteria for a positive MAT are a four-fold increase in antibody titre, or a conversion from seronegativity to a titre of 1/100 or 1/200 (18, 35).

This test is a very sensitive, high specific and reliable assay when used by skilled personnel, but preparations for MAT require meticulous culture of a collection of the strains used as antigen suspensions in the tests (34). For optimum sensitivity, it should use antigens representative of all the serogroups known to exist in the region (36). Since it requires the use of live organisms as antigen, it poses a danger to laboratory personnel and potentially biohazardous. Their regular subculture and quality control for authenticity, purity agglutination and maintenance of several leptospiral serovars may be difficult, tedious, expensive, and time consuming. Moreover it cannot be standardized as live antigens are often used and various factors, such as the age and density of the antigen culture can influence the agglutination titre (36, 37).

In comparison with other serological tests, the MAT is considered the “gold standard” of serodiagnosis tests because of its unsurpassed diagnostic (serovar/serogroup) specificity (38, 39).

2.3.2. Enzyme-Linked Immunosorbent Assay Test

Enzyme-linked immunosorbent assay (ELISA) has been reported to be more sensitive than the conventional serological tests for diagnosis of leptospirosis (20, 40). Recently, this test is widely used as a genus specific screening test. The use of computer assisted automated readers and the appropriate controls improve the reproducibility and predictive value of this test (31).

Enzyme-linked immunosorbent assay is a genus-specific test which can detect IgM-class antibody (sometimes also IgG antibody) in the early phase of the disease so that recent leptospirosis may be indicated. The IgM may remain detectable for several years. The cut-off point (positive titre) is best determined on the basis of the same considerations as presented above for the MAT. Genus-specific tests tend to be positive earlier in the disease than the MAT. However, the ELISA test does not give an indication of the infecting serovar (34, 41, 42). Where no antibody is detected or only a low ELISA titre is found, a second serum sample should be examined for seroconversion or a significant rise in titre (41, 42).

Enzyme-linked immunosorbent assays for detection of anti-leptospiral antibodies have been developed based on detection of antibodies against surface proteins or lipoproteins of *Leptospira*. Earlier, most of the research on leptospiral antigens was focused on lipopolysaccharides (LPS) which have been identified as an immunodominant antigen, but LPS antigens vary greatly among different leptospiral serovars. In contrast to LPS, leptospiral membrane proteins are thought to be highly conserved and expressed during infection (41, 43-45).

The outer membrane protein genes such as *lipL32*, *ompL1*, *lipL41*, *ligB*, *lipL21* genes are highly conserved sequences among pathogenic Leptospires and may be a suitable candidate as recombinant antigen for developing serodiagnostic test such as ELISA (44, 46-51).

Recombinant protein based ELISA is a suitable and safe procedure for the examination of a large number of sera that requiring a small amount of serum, and diagnosis can be made during the early phase of the disease (34, 52, 53).

ELISA can detect IgM-class antibody in the early phase of the disease so that current or recent infection may be indicated. Where no antibody is detected or only a low ELISA titre is found, a second serum sample should be examined for seroconversion or a significant rise in titre (41, 52).

Some ELISA test systems are less specific than the MAT and weak cross reactions due to the presence of other diseases may be observed. Therefore, the results should be confirmed by the MAT (39).

2.3.3. Latex Agglutination Test

This test is simple and rapid technique. Antiserum will react with the leptospiral antigen to cause agglutination of the particles. Antigen prepared from *L. biflexa* serovar Patoc will cross-react with human convalescent sera to provide a useful screening procedure. This test depends on the sensitization of commercially available latex particles with a leptospiral antigen (54, 55).

2.3.4. Lepto-Dipstick Test

This test is used for the detection of *Leptospira*-specific IgM antibodies in human sera. Heat stable antigen which prepare from *Leptospira biflexa* serovar Patoc and coat onto the lower band and internal control set up in the upper band, detection agent also incorporated. The sensitivity and specificity of this method appeared acceptable. The dipstick assay revealed cross-reactivity with sera from patients with HIV, hantavirus, toxoplasma infection, Lyme borreliosis, malaria, meningococcal meningitis and hepatitis A infection. In contrast, no cross-reactivity was observed with these sera in IgM ELISA. The highly stable reagents and simple implementation makes this method suitable for use in clinical and field laboratories in tropical countries (19, 56).

2.4. Molecular Diagnosis of Leptospirosis

2.4.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method of amplifying specific segments of leptospiral DNA in clinical samples such as blood or urine, cerebrospinal fluid and tissue samples (ante or postmortem), to detectable levels. Thus, the presence of pathogenic leptospires is confirmed by identifying specific segments of leptospiral DNA (57, 58). In addition, subsequent or concomitant hybridization with labeled probes and/or performed with a nested primer set makes a highly specific method (59).

It is a rapid, sensitive and specific means of detecting leptospiral infection, in contrast to serology tests. Further benefit is the ability to identify early infection especially during the first few days of the disease even before antibodies are detectable (34).

Numerous studies confirmed its high specificity and sensitivity, with the capability of detecting as few as 10 organisms in a sample(46, 47, 49, 50, 60). Leptospiral DNA has been detected in body where antibody titres may be lower and appear later than in serum (34).

However, PCR requires special equipment and dedicated laboratory space, and also skilled personnel. It may also give false-positive results in the presence of minute amounts of extraneous DNA that may contaminate working areas. It may also give false-negative results because inhibitors are present in the clinical materials that are being examined (61).

A fundamental question for efficient molecular diagnostic development will be to address which genes are expressed during infection (62).

To date, many PCR methods have been described (58, 60, 63). As a major target, the *rrs* gene encoding 16S rRNA has been used (64, 65). Other targets include the *secY* and *flaB* genes for the combined primer set of G1/G2 and B64I/B64II, respectively) (58, 66).

Recently, the leptospiral antigens that express during infection have potentially important implications in the development of molecular diagnostic methods (67). So, an important focus of the current leptospiral research is mainly on the OMPs (68, 69). The OMPs that are exposed on the leptospiral surface are potentially relevant in virulence pathogenesis because of their location at the interface between leptospires and the mammalian host (70). The OMPs genes such as *lipL32*, *ompL1*, *lipL41*, *ligB*, and *lipL21* are present in all pathogenic serovars while not in saprophyte serovars. These genes are highly conserved among various pathogenic *Leptospira* species. Therefore, these genes are useful for detection of leptospiral infection and suitable for designing of positive control in PCR assay (46-50). Although PCR is now widely used for the diagnosis of many diseases, its general value for the rapid diagnosis of leptospirosis has not been evaluated worldwide as it is not yet widely used, particularly in tropical and subtropical countries.

2.4.2. Real-Time Polymerase Chain Reaction

Identification of real-time PCR technology is a relatively new identification method (71). This method has the ability to distinguish between pathogenic leptospires using melting curves and gives a result much quicker than conventional PCR and is less prone to contamination. Thus, it provides a fast alternative for diagnosis of *Leptospira* spp (72, 73).

Recently, a number of real-time PCRs were introduced as a rapid and sensitive and specific tool for leptospires detection, reducing the risk of false positive results by carryover contamination. Polymerase chain reactions targeting the *ligA*, *ligB* genes (74), *rrs* gene (75), and *lipL32* (72, 76) are claimed to be specific for pathogenic leptospires and therefore appropriate for diagnostic purposes (77).

3. Conclusions

Leptospirosis present with a wide variety of clinical manifestations; therefore, the diagnosis should be confirmed by laboratory tests. Laboratory support is also necessary for epidemiological study in community. An ideal test will need to discriminate between leptospirosis and a broad spectrum of diseases that cause acute febrile illness and have overlapping clinical presentations.

The disease is usually diagnosed in the laboratory by detecting antibodies (serodiagnosis), culturing the bacteria from blood, urine or tissues, demonstrating the presence of leptospires in urine and tissues, or molecular methods (e.g. PCR) may be available in some centers. Choice of test

for diagnosis of leptospirosis depends on the stage of the disease.

Isolation of pathogenic leptospires is the only definitive proof of disease. However, *Leptospira* is fastidious and its isolation from clinical samples is laborious and requires both special media and incubates for several weeks.

Although detection of antibodies is by itself no proof of a current infection, serological methods are often the most appropriate diagnostic methods. Recently, two tests are used for the serological diagnosis of leptospirosis, which includes MAT and ELISA.

The major advantage of the MAT is its high specificity. An important disadvantage is the need for facilities to culture and maintain panels of live leptospires. Furthermore, the test is technically demanding, potentially biohazardous and time consuming. Moreover, it cannot be standardized as live antigens are often used. Despite the drawbacks of the MAT, it still is a standard serology test for detection of leptospires because of its incomparable diagnostic specificity (serovar/serogroup) in comparison with other currently available tests; however, its use is restricted to reference laboratories. It is clear that ELISA (genus-specific test) is more sensitive than live antigen MAT, but lack the serovar specificity of the MAT. Also, in contrast to the MAT, ELISA can be standardized and test tends to be positive earlier in the disease.

Polymerase chain reaction is a rapid, sensitive and specific possible method of early diagnosis of leptospirosis, when bacteria may be present and before antibody titres are at detectable levels. Also, PCR should preferably be combined with a hybridization step and/or performed with a nested primer set to ensure high specificity.

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