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REVIEW ARTICLE

Laboratory diagnosis of leptospirosis: A challenge

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Leptospirosis is caused by pathogenic bacteria called leptospire that are transmitted directly or indirectly from animals to humans. It occurs worldwide but is most common in tropical and sub-tropical areas. It is a potentially serious but treatable disease. Its symptoms may mimic those of a number of other unrelated infections such as influenza, meningitis, hepatitis, dengue, or other viral hemorrhagic fevers. The spectrum of the disease is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality. Laboratory diagnosis tests are not always available, especially in developing countries. Numerous tests have been developed, but availability of appropriate laboratory support is still a problem. Direct observation of leptospire by darkfield microscopy is unreliable and not recommended. Isolation of leptospire can take up to months and does not contribute to early diagnosis. Diagnosis is usually performed by serology; enzyme-linked immunosorbent assay and the microscopic agglutination tests are the laboratory methods generally used, rapid tests are also available. Limitation of serology is that antibodies are lacking at the acute phase of the disease. In recent years, several real-time polymerase chain reaction assays have been described. These can confirm the diagnosis in the early phase of the disease prior to antibody titers are at detectable levels, but molecular testing is not available in restricted resources areas.

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Introduction

The clinical presentation of leptospirosis is unspecific, misdiagnosis is frequent, and diagnosis is based upon laboratory results. The laboratory diagnosis of leptospirosis is challenging. The only sensitive and specific test accurate at

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the acute phase of the disease is polymerase chain reaction (PCR), which is not available in most high endemic areas and the serological reference method by micro agglutination testing (MAT) is restricted to reference laboratories. In this review we present the advantages and disadvantages of the laboratory methods for leptospirosis diagnosis and we focus on the rapid tests currently used in countries with low resources.

History

Adolf Weil reported the syndrome of icteric leptospirosis with renal failure in 1886 but the disease was recognized earlier as an occupational hazard of rice harvesting in ancient China.^{1,2} Leptospire were first visualized in autopsy specimen from a patient thought to have had yellow fever. The role of the rat as a source of human infection was discovered in 1917.

Bacteriology

Leptospire belong to the order Spirochaetales, family *Leptospiraceae*, genus *Leptospira*.³ They can be pathogenic or saprophytic. Pathogenic leptospire can be maintained in nature in the renal tubules of animals and saprophytic leptospire in many types of wet or humid environments.

Classification of leptospire

Prior to 1989, the genus *Leptospira* was divided into two species: *Leptospira interrogans* (pathogenic strains) and *Leptospira biflexa* (saprophytic strains). The species were divided into serovars and the serovars grouped into serogroups. More than 24 serogroups and 250 serovars of pathogenic leptospire have been described to date.⁴ The serovar concept has been widely accepted because it has some epidemiologic value, but it has no taxonomic standing.⁵

The serologic classification has been replaced by a genotypic one. The genomospecies include all *L. interrogans* and *L. biflexa* serovars. The genus *Leptospira* is divided into 20 species classified into saprophytic, intermediate, and pathogenic groups. The genomospecies of *Leptospira* do not correspond to the previous species *L. interrogans* and *L. biflexa*.

Epidemiology

Leptospirosis is the most widespread zoonosis in the world and is considered as an emerging global public health disease.⁶

It occurs worldwide with a higher incidence in warm than in temperate regions. The number of severe human cases worldwide is estimated above 500,000.⁷ Incidences range from 0.1–1/100,000/year in temperate climates, 10–100/100,000/year in the humid tropics to over 100/100,000/year during outbreaks and in high-exposure risk groups. The endemicity of the disease is mainly located in the Caribbean, Central and South America, Southeast Asia and Oceania.⁸ During the past several years, large outbreaks have occurred in many countries, particularly in

Southeast Asia, Central and South America. Case-fatality rates range from <5% to 30%.

Effective surveillance systems with appropriate laboratory support exist in developed countries but are often lacking in the disease-endemic developing areas. The reported incidence of leptospirosis reflects the availability of laboratory diagnosis and the clinical index of suspicion as much as the incidence of the disease. For example, the actual incidence of leptospirosis in the Asia Pacific region is not well documented⁹ and leptospirosis is often underestimated.¹⁰ With the hyperendemic Southeast Asia zone, Oceania exhibits a significant burden of leptospirosis. In the Asia Pacific region, predominantly in developing countries, leptospirosis is largely a water-borne disease.

Numerous animals, primarily mammals, are sources of human infection. Rodents are the most important and widely distributed reservoirs of leptospire. Some serovars are associated with a particular species of natural maintenance host. In chronic infections, leptospire are localized in the kidneys, usually without detectable clinical manifestations.

The usual mode of contamination is abrasions or cuts in the skin or via the conjunctiva through direct or indirect contact with urine or tissues of infected animals. Other modes of contamination, such as inhalation of water or aerosols, animal bites, or interhuman transmission, have been rarely demonstrated.

Leptospirosis is an occupational disease for veterinarians, farmers, abattoir workers, butchers, hunters, rodent control workers, and other occupations requiring contact with animals. Indirect contact with contaminated wet soil or water is responsible for the great majority of cases in the tropics, either through occupational exposure as in rice or taro farming, flooding after heavy rains, or exposure to damp soil and water during avocational activities. Contamination due to recreational exposures is increasing, often in association with adventure tourism in tropical endemic areas.

Three epidemiological patterns have been defined: in temperate climates where few serovars are involved and human infection occurs by direct contact with infected animals; in tropical wet areas where there are many more serovars infecting humans and animals and larger numbers of reservoir species; and in the urban environment as a rodent-borne infection.¹¹

Clinical presentation

It may range from a flu-like illness to a serious and sometimes fatal disease. Confusion with other diseases, especially dengue fever and other hemorrhagic fevers, is frequent in the tropical areas. The mean incubation time is 1–2 weeks, with a range of 2 days to 30 days. The acute or septicemic phase lasting about 1 week is followed by an immune phase characterized by antibody production. The great majority of infections are subclinical or of very mild severity. The most common symptoms are febrile illness of sudden onset, chills, headache, myalgia, abdominal pain, and conjunctival suffusion. Other clinical presentations may be predominant, in addition to hepatic or renal dysfunction, leptospirosis should be seriously considered in

patients with pulmonary symptoms and fever, especially in subtropical and tropical areas, as reported in a retrospective study conducted in Taiwan.¹²

Laboratory diagnosis

Specimen collection

Several blood tubes should be collected at the early phase of the disease: standard blood culture bottle or tube; nonadditive or gel separator tubes for chemistry and serology; and EDTA tube for blood count.

For blood culture, blood with heparin to prevent clotting is recommended but ideally blood is inoculated directly into blood culture bottles containing culture medium for leptospires.

For molecular testing, published studies showed mixed results¹³: serum was reported to be inferior to plasma^{14,15}; serum was reported to be superior to whole blood; and buffy coat was reported to be superior to plasma and serum.¹⁶ Heparin was reported to be inhibitory.¹⁷

All blood samples must be conserved for subsequent additional testing. Acute blood samples are of great importance for serology in order to demonstrate a seroconversion.

Nonspecific laboratory findings

The various nondiagnostic abnormalities are reported in Table 1¹⁸; these can only suggest leptospirosis. Specific microbiological tests are required for confirmation.

Microscopic demonstration

Leptospires cannot be observed under the ordinary light microscope but by darkfield microscopy as thin, coiled, and

rapidly moving microorganisms. Sensitivity of darkfield microscopy is approximately 10^7 leptospires/L. Direct examination of blood and urine has both low sensitivity and specificity, it is subject to misinterpretation of fibrin or protein threads, then is not recommended as a routine procedure.

Leptospires are not stained by conventional Gram staining. Available staining methods to increase the sensitivity of direct examination are: immunofluorescence, immunoperoxidase, silver staining, Warthin-Starry staining, immunohistochemistry, and *in situ* hybridization. All of these suffer from the same drawbacks as darkfield microscopy: a high risk of false-positive and false-negative results.

Isolation of leptospires

Samples for culture should be collected prior to the administration of antibiotics. Blood, cerebrospinal fluid and dialysate should be cultured in the first 10 days of the illness, and urine from the second week of the illness. Several specific media were described by Fletcher et al. The most used medium is based on the oleic acid-albumin Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton Dickinson and Company, Difco™) and is available commercially.

Samples should be stored and transported at ambient temperatures. Survival of leptospires in human urine is limited so urine should be processed immediately. Cultures are incubated in the dark at 28–30°C and examined weekly by darkfield microscopy for up to 13 weeks prior to being discarded.

Antigen detection

Different antigen detection tests have been developed but none of them is sensitive enough to be routinely used.¹⁹

Antibody detection

The MAT, which is the serological reference test, was first described in 1918 by Martin and Pettit. Live antigens representing different serogroups are reacted with serum samples and the agglutination is examined by darkfield microscopy. Panels of live leptospires belonging to different serovars must be maintained in the laboratory. As a minimum, the panel should include all locally circulating serovars and, if these serovars are unknown or subject to change, the panel should include serovars representing all serogroups. An incomplete panel should be responsible for false negative results. MAT may be positive from Day 10–12 after the onset of illness, sometimes later if specific antibiotics have been prescribed. MAT was reported to have a sensitivity of 41% during the 1st week, 82% during the 2nd to 4th week, and 96% beyond the 4th week of illness.²⁰ The cut off value on a single sera depends from the seroprevalence. For the Center for Disease Control, a probable case is defined as a titer ≥ 200 associated with a clinically compatible illness²¹; in a publication from the Center for Disease Control of Taiwan, an antibody titer ≥ 100 was regarded as a probable case of leptospirosis²²; in a study

Table 1 Nonspecific laboratory findings

1. Blood analysis
1.1. Leukocytosis with a shift to the left
1.2. Thrombocytopenia in $>50\%$ ¹⁸
1.3. Elevated
1.3.1. Erythrocyte sedimentation rate
1.3.2. Creatinine (usually <20 – 80 mg/L)
1.3.3. Urea (usually <1000 mg/L)
1.3.4. Aminotransferases (rarely > 200 IU/L)
1.3.5. Bilirubin (may rise to 800 mg/L)
1.3.6. Alkaline phosphatase
2. Urine analysis
2.1. Proteinuria, pyuria, microscopic hematuria, hyaline, and granular casts
3. Cerebrospinal fluid analysis
3.1. Normal or slightly elevated cerebrospinal fluid pressure
3.2. Initially a predominance of polymorphs or lymphocytes (total cell counts generally $<500 \times 10^6$ /L) and lymphocytes predominance later. Pleocytosis can persist for weeks
3.3. Elevated protein (50 – 100 g/L)
3.4. Glucose is usually normal
3.5. Xanthochromia may occur

conducted in Thailand a positive MAT was defined as a single titer ≥ 400 .²³ The Leptospirosis Burden Epidemiology Reference Group consider a single MAT $\geq 1:400$ (or single MAT $\geq 1:100$ in nonendemic regions) to be consistent with leptospirosis. A low titer is appropriate in a population in which exposure to leptospirosis is uncommon but, if exposure is frequent, as in most tropical countries, a higher cut-off titer is necessary. In very high endemic areas, a single titer of 800 in symptomatic patients is generally indicative but a 1600 titer has been recommended. In cases of previous infection with a different serogroup, interpretation is complicated by the "anamnesic response" (the rise in antibody titer is directed against a previous infecting serovar). A fourfold or greater rise in titer between paired sera is required to confirm leptospirosis. MAT detects both class M and class G antibodies, and cannot differentiate between current, recent, or past infections. It may identify the presumptive serogroup, and under the best conditions the serovar because interpretation is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples.

The MAT is complex to control and perform; it cannot be standardized because live leptospires are used as antigens.

Enzyme-linked immunosorbent assay (ELISA) detects antibodies reacting with a broadly reactive genus-specific antigen and thus is not suitable for identification of the causative serovar or serogroup. Commercial kits are available. The cut-off point is determined on the same considerations as for the MAT. Serogroups *Grippityphosa* and *Australis* gave false negative results. ELISA is usually positive from Day 6–8, earlier than the MAT, and it may be negative earlier. Most of the commercial ELISA kits use as antigen the nonpathogenic *Leptospira biflexa patoc* strain. ELISA allows detection of specific IgM class antibodies. IgM may remain detectable for several months or even years. Positive ELISA should be confirmed by MAT.

Other serological tests have been developed: complement fixation, counterimmunoelectrophoresis, indirect fluorescent antibody, indirect hemagglutination (IHA), sensitized erythrocyte lysis, latex agglutination (LA), macroscopic slide agglutination, microcapsule agglutination, and Patoc slide agglutination.

Rapid screening tests based on four immunological principles are used: particle agglutination (centrifugation of whole blood required, detection of a weak agglutination is difficult, reagents often require refrigeration); immunodot or dipstick/comb (results are visualized as a spot, dot, or line, test requires less than 30 minutes to develop, reagents do not require refrigeration); immunofiltration or flow-through device (the assay require several steps, reagents often require refrigeration); immunochromatography or lateral flow (a visible line at test and control location indicates a positive reaction, no special equipment required, they are one step tests and are completed within 15 minutes; depending on the assay, whole blood, serum, or plasma can be used, reagents do not require refrigeration).²⁴

Evaluation of rapid screening tests

Rapid tests are easy to use and can be performed by individuals without special technical training. Some of them

can be performed on whole blood and can be stored for prolonged periods at ambient temperatures, and standard laboratory equipment is not required. Even though the reading and interpretation of rapid test reactions is claimed to be simple, some training is required to perform and interpret them correctly. Interobserver variability in reading and interpretation of the end points may provide inconsistent results.

These tests are primarily IgM detection assays, but because IgM is not detectable until the second week after symptom onset, they have low sensitivity in the early acute phase of illness when patients present for medical treatment.²⁵

In a large multicenter evaluation of an IgM *Leptospira* dipstick assay conducted in areas with high and low leptospirosis endemicity, the mean sensitivity was 60.1% on sera collected within the first 10 days of the illness and the results were concordant with an ELISA IgM.²⁶

Four rapid tests (ELISA IgM, IHA, IgM dipstick assay, IgM dot-ELISA dipstick test) were evaluated: the sensitivity ranged from 38.5% (IHA) to 52.7% (IgM dipstick assay) on acute sera collected prior to 14 days after onset of the disease, by comparison, the sensitivity was 48.7% with MAT.²⁷ Sensitivity on convalescent sera ranged from 67.2% to 84.4% and was 93.8% for MAT.

Eight rapid tests (IHA, 2 IgM dipstick assay; indirect fluorescent antibody, 3 ELISA IgM, LA) have been evaluated in Hawaii and the authors concluded that all tests were insensitive for diagnosis within the first week of the disease while it is during this time that important therapeutic decisions are likely to be made.²⁸ Evaluation of two rapid tests at the acute visit for leptospirosis (IgM dipstick assay, LA) and dengue (IgM dipstick assay, Dengue duo rapid strip) in a tropical field setting yielded sensitivity from 13% to 22% for leptospirosis (positive predictive value range, 15–18%) and from 8% to 19% for dengue with the conclusion that their utility at the acute phase of dengue and leptospirosis is limited.²⁹

Because of their low sensitivities, use of these tests for the initial management of acute mild leptospirosis in adults was inferior to empirical treatment in a study conducted in Thailand.³⁰

The low sensitivity of these tests at the acute phase of the disease is not related to the rapid test format but is due to the fact that the tests detect IgM antibodies.

Molecular diagnosis

The need for rapid diagnostics at the time of admission has led to the development of numerous PCR assays. Their advantage lies in the ability to obtain a definitive diagnosis during the acute stage of the illness prior to antibodies are detectable, while treatment may be effective.

PCR detects DNA in blood in the first 5–10 days after the onset of the disease and up to the 15th day. The bacterial load in serum/blood ranges from 10^5 to 10^9 leptospires/L.

PCR allows detection of leptospires in culture negative blood if the patient has received an effective antimicrobial drug but have not cleared nonviable organism.³¹

PCR is based on the detection of genes universally present in bacteria as *gyrB*,³² *rrs* (16S rRNA gene),³³ *secY*³⁴; or

genes restricted to pathogenic *Leptospira* spp. as *lipL32*, *lfb1*,³⁵ *ligA*, and *ligB2*.³⁶

Conventional PCR assays have not been well evaluated, leaving its diagnosis value unclear.^{37,38} It has been replaced by real-time quantitative PCR (qPCR), which combines amplification and detection of amplified product in the same reaction vessel with excellent sensitivity and specificity and low contamination risk.³⁹ Detection can be performed using SYBR Green, which provides sensitive detection but is less specific than detection using fluorescent probe technology such as TaqMan probes.

A number of qPCRs have been introduced: SYBR Green qPCR targeting *secY* or *lipL32*; TaqMan qPCR targeting *lipL32*; *rss* (16S); and a multiplex assay for simultaneous detection and differentiation of pathogenic and nonpathogenic leptospires.⁴⁰

Four qPCR, SYBR green, and TaqMan assays targeting the *secY*, *lfb1*, and *lipL32* genes have been recently evaluated. They detected from 10⁵ bacteria/L to 10⁶ bacteria/L of pure culture, whole blood, plasma, and serum samples. The authors recommend a continual evaluation and, if necessary, modification of the primers and/or probes used to ensure effective detection of the circulating leptospires isolates. Lyophilized reagent-based PCR assay for the detection of leptospires have been developed.⁴¹

Typing methods

Severe cases can be due to all infective serovar. Identification is not required for clinical care but is of particular interest from the public health perspective. It may indicate the sources of infection and reservoirs and thus contribute to the choice of methods for prevention and control.

Antigen–antibody reactions, such as MAT, can be used to identify strains, but are laborious and time-consuming, which restricts their use to specialized laboratories. In serogroup determination the antigen suspension of the unknown strain is used in titrations with several antisera representing all recognized serogroups; in the cross-agglutination-absorption test, the reaction of the unknown strain and its antiserum is compared with reference strains and their antisera, typing by monoclonal antibodies is based on the recognition of antigen patterns of serovars by panels of monoclonal antibodies.

As serotyping is complex and can only be performed in reference laboratories, a number of molecular techniques have been developed as alternatives to or in complement to serotyping including: DNA–DNA hybridization, restriction fragment length polymorphisms, pulsed-field gel electrophoresis, ribotyping, PCR-based typing, insertion sequences based typing, amplification with specific primers, variable number of tandem repeats, low-stringency single specific primer PCR, PCR restriction endonuclease analysis, arbitrarily primed multiple locus sequence typing, random amplification of polymorphic DNA, and determination of sequences of PCR products.⁴²

The usual target for sequence-based identification of *Leptospira* species is the 16S rRNA gene.⁴³ Other genes can be used, such as *rpoB* encoding the β -subunit of RNA polymerase^{44–46} or *gyrB* encoding the β -subunit of DNA gyrase.

Susceptibility testing

Susceptibility testing is not routinely performed due to the long incubation time required and the difficulty in quantifying growth accurately.

Safety procedure

Standard microbiological laboratory safety procedures are required when working with leptospires (Biosafety Level II facilities).

Definition of leptospirosis

The Leptospirosis Burden Epidemiology Reference Group definitions of leptospirosis are reported in Table 2.

The most relevant tests

Laboratory testing depends on the temporal stage of the disease, its prevalence, the presence of a laboratory and if present the availability of specific tests. Advantages and disadvantages of common diagnostic tests for leptospirosis are reported in Table 3.

Within the first days of the disease, the only sensitive and specific test is PCR. At this stage, rapid diagnosis is only possible if quick and easy molecular testing is possible. In resource-restricted countries, the cost and requirement for special equipment and technical expertise remain as barriers limiting its use. As in most endemic area, molecular testing is not available in general practice, confirmation of the diagnosis cannot be obtained rapidly.

From the second week on the disease, serological diagnosis is based on the detection of specific IgM. If a laboratory is present, all serological tests can be performed, except MAT; in the absence of laboratory, rapid tests can be performed.

Table 2 Leptospirosis Burden Epidemiology Reference Group definitions of leptospirosis

1. Definitive case: symptoms consistent with leptospirosis and any one of the following:
 - 1.1. 4-fold increase in MAT titer between acute and convalescent serum samples
 - 1.2. Single MAT $\geq 1:400$ (or single MAT $\geq 1:100$ in nonendemic regions)
 - 1.3. Isolation of *Leptospira* spp. from a normally sterile site
 - 1.4. Detection of *Leptospira* spp. in clinical samples using histological, histochemical, or immunostaining techniques
 - 1.5. *Leptospira* DNA detected by PCR
2. Presumptive case: symptoms consistent with leptospirosis and any one of the following:
 - 2.1. Presence of IgM antibodies, as shown by ELISA or dipstick
 - 2.2. Presence of IgM or IgG antibodies, as shown by immunofluorescence assay

Table 3 Advantages and disadvantages of common diagnostic tests for leptospirosis

	Microscopic demonstration	Culture	Serology MAT	Serology ELISA IgM	Serology rapid tests IgM	Molecular testing
Specimen collection	Blood, urine, CSF	Blood, urine, CSF, tissues	Blood	Blood	Blood	Blood, urine, CSF, tissues
Window of positivity	1 st wk: blood, CSF 2 nd wk: urine	1 st 10 d	From Day 10–12	From Day 6–8	From Day 6–8	From Day 5–10 in blood
Processing time	Available in 1 h	2 wk to 4 mo	Several wks if not locally available	Available in 1 d	Available in 15–30 min	Available in 1 d
Early diagnosis	No	No	No	No	No	Yes
Definitive diagnosis if positive	No	Yes	Yes (seroconversion)	Yes (seroconversion)	Yes (seroconversion)	Yes
Identification	No	Yes (if MAT or molecular testing available)	Yes	No	No	Yes (by additional molecular tests)
Remark	Low sensitivity and specificity Not recommended for diagnosis	Low sensitivity, slow, difficult	Gold standard but very difficult	Needs confirmation by MAT	Needs confirmation by MAT	The only sensitive test at the acute phase
Equipment required	Dark field microscope	Specific culture media	Reference laboratory only	Standard laboratory	Laboratory equipment not required	Special equipment, dedicated laboratory space, highly skilled personnel

If the time from the onset of the illness is not indicated, we strongly suggest that the laboratory contacts the physician in order to perform the most relevant test: in the 1st week the negative predictive value of serology is very low, and from the 2nd week the negative predictive value of PCR is very low. In our laboratory we perform both tests, and despite frequent information of the clinicians, inappropriate tests are prescribed and are responsible for misdiagnosis.

The low level of concordance between PCR, MAT, and ELISA IgM reflects the phases of the disease suggesting that molecular and serological methods may be used in different periods.⁴⁷

Treatment should be initiated as soon as the diagnosis of leptospirosis is suspected and preferably prior to the fifth day after the onset of illness. Clinicians should not wait for the results of laboratory tests prior to starting treatment.

Even tests with high sensitivity and specificity may have limited utility in general use because of low predictive values. The predictive value of a test varies with the prevalence of the disease in the target population. The positive predictive value of a rapid diagnostic test for leptospirosis was poor at both acute and convalescent visits because of the low prevalence of the disease in the population of febrile patients in Thailand.

In many developing countries, including most of the leptospirosis endemic areas, laboratory capabilities to detect pathogenic microorganisms are often inadequate. Sometimes, basic necessities and equipment are missing such as electricity, refrigerators, and trained laboratory personnel. Because of their ease of use, even in primary health centers, rapid tests are often used in routine practice in many clinical settings. However, these rapid diagnostic tests may not reach optimal sensitivity until at least a week after onset of fever, well after the time when patients first present to medical care. As the sensitivity of the tests is low at the acute visit, these rapid diagnostic tests should be used with caution to rule out leptospirosis, the same restriction should be considered when using ELISA IgM tests.

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