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REVIEW ARTICLE

LABORATORY DIAGNOSIS OF LEPTOSPIROSIS: A REVIEW

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ABSTRACT: Leptospirosis is a zoonosis of global distribution caused by infection with pathogenic spirochaetes of the genus Leptospira. Humans are accidental hosts and usually become infected through contact with water or soil contaminated by the urine of infected animals such as rodents, dogs, cattle and pigs. In developing countries such as India, leptospirosis is often underdiagnosed because of its protean clinical manifestations leading to significant morbidity and mortality. It occurs as a self-limited illness in 85% to 90% of the cases and icteric leptospirosis or Weil's syndrome, a more serious, potentially fatal syndrome which occurs in 5% to 10% of the cases. Microbiological diagnosis of leptospirosis aims at demonstrating the leptospires, by culturing them or by demonstrating an appreciable antibody response to them. A definite diagnosis of leptospirosis is based either on isolation of the organism from the patient or on seroconversion or a rise in antibody titre in the MAT. Leptospires may be visualized in clinical material by DGM or by IF or light microscopy after appropriate staining. The sensitivity of blood cultures is low; hence culture is primarily used for retrospective diagnosis. There are numerous serological tests available for diagnosis of leptospirosis like Macroscopic agglutination test (MSAT), Indirect fluorescent antibody test (IFAT), Sensitised erythrocyte lysis test (SEL), Complement fixation test (CFT), Enzyme Linked Sorbent Assay (ELISA), Microcapsule agglutination test (MCAT), Lepto-Dipstick, Latex agglutination test, Dried Latex agglutination test (Lepto Tek Dri-Dot), but they are only genus specific. To identify the specific serovar Microscopic agglutination test (MAT) or culture has to be done. The various available options for a diagnosis of leptospirosis have been explored in this article. A thorough literary search was done in the various published data available- pub med search was done as well as published books were also surveyed on the same topic.

KEYWORDS: Leptospira, Leptospirosis, Weil`s disease, Dark field microscopy, Microscopic Agglutination Test (MAT).

INTRODUCTION: Leptospirosis is a zoonosis of global distribution caused by infection with pathogenic spirochaetes of the genus Leptospira.[1] The etiologic agent is Leptospira interrogans.[2]

Leptospirosis is now identified as one of the emerging infectious diseases.[3] It is also recognized as the most common zoonotic infection in the world.[4] The incidence is higher in the tropics.[5] Large number of outbreaks have been reported from several countries in recent times, namely Latin America, Caribbean, Nicaragua, Brazil, Southeast Asia, USA, Malaysia and India.[4] Leptospirosis has been known as "Weil`s disease", "Rice field fever", "Swamp fever", "Flood fever", "Canicola fever", " Spirochaetal jaundice" etc.[6]

Humans are accidental hosts and usually become infected through contact with water or soil contaminated by the urine of infected animals such as rodents, dogs, cattle and pigs. Exposure of skin or mucous membranes to leptospires can lead to infection.[7] The infection in human beings varies greatly in severity from the mildest form of nonspecific febrile illness to the severe, fatal form with hepatorenal failure named as "Weil`s disease".[8]

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In developing countries such as India, leptospirosis is often underdiagnosed because of its protean clinical manifestations leading to significant morbidity and mortality.[9]

A literary search was done in the various published data available and pubmed search was done on the topic. The various available options for a diagnosis of leptospirosis have been explored in this article.

Laboratory Diagnosis: *Leptospira* consist of a group of pathogenic leptospires, *Leptospira interrogans* sensu lato and non-pathogenic leptospires, *Leptospira biflexa* sensu lato[10] within each species, large number of serovars are differentiated using polyclonal agglutinating antibodies. More than 250 serovars of pathogenic leptospires have been described. Because of the large number of serovars, antigenically related serovars are grouped into serogroups, for convenience in serologic testing.

The phenotypic classification of leptospires has been replaced by a genotypic one. DNA hybridization studies led to the definition of 10 genomospecies of Leptospira. Centre for Disease Control (CDC) recently defined 16 genomospecies of Leptospira.[1]

The incubation period for leptospirosis is usually 7 to 12 days. It occurs as two clinically recognizable syndromes- most common is anicteric leptospirosis, a self-limited illness that occurs in 85% to 90% of the cases and icteric leptospirosis or Weil's syndrome, a more serious, potentially fatal syndrome which occurs in 5% to 10% of the cases.[2] After the incubation period, an acute leptospiraemic phase is followed by an immune phase.[11]

In anicteric disease, Liver function tests show slight elevation in aminotransferases, bilirubin and alkaline phosphatase in the absence of jaundice. In icteric leptospirosis, liver function tests generally show a significant rise in bilirubin, with lesser increase in transaminases and marginal increase in alkaline phosphatase levels. Renal function impairment is indicated by raised plasma creatinine levels.[3] Other laboratory abnormalities include anaemia, thrombocytopenia, leucocytosis with neutrophilia and an increase in the level of creatinine phosphokinase.

Microbiological diagnosis of leptospirosis aims at demonstrating the leptospires, by culturing them or by demonstrating an appreciable antibody response to them.[2] A definite diagnosis of leptospirosis is based either on isolation of the organism from the patient or on seroconversion or a rise in antibody titre in the MAT.[11] The first week of illness diagnostic tests consist of blood culture, examination of sera collected during first and third week for evidence of rising titre and culture of urine after the third week of illness.[6]

A) Direct Evidence: **1. Demonstration of leptospires or their products:**
Dark-field microscopy.
Phase contrast microscopy.
Silver staining.
Immunofluorescence.
Immunoperoxidase.
DNA hybridization.
Polymerase chain reaction.

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2. Isolation of leptospires:
Blood.
Urine.
CSF.
Body fluids and tissues.
B) Indirect Evidence:
1. Detection of antibodies to Leptospira:
i. Genus specific tests:
Macroscopic agglutination test.
Indirect fluorescent antibody test.
Sensitised erythrocyte lysis test.
Complement fixation test.
Enzyme Linked Immuno Sorbent Assay.
Microcapsule agglutination test.
Lepto-Dipstick.
Latex agglutination test.
Dried Latex agglutination test (Lepto Tek Dri-Dot).
ii. Serogroup/serovar specific tests:
Microscopic agglutination test.
Serovar specific ELISA.

Table 1: Tests for the diagnosis of Leptospirosis

Direct Evidence: Dark-Field Microscopy: Microscopy of blood is of value only during the first few days of the acute illness. Dark field microscopy (DFM) examination of body fluids such as blood, urine and CSF has been used. Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by DFM.[3] It is a simple method, but may not be positive if there are few bacteria in the sample. Double centrifugation of the sample at low speed to separate the cellular elements, and then at high speed, help concentrate the leptospires.[2] It is the procedure of choice for the demonstration of the organisms in tissue fluids.[12] DFM, after differential centrifugation of Ruys, may enhance the chances of seeing leptospires and thereby make an early diagnosis possible.[13] Blood or spinal fluid may be examined during the first week, of illness. Urine can be examined from the end of the second week, till about 40 days.[14]

It is technically demanding. Recognizing leptospires is difficult, particularly when only small numbers are present.[10] At best, DFM should be considered as an aid that may suggest but not establish a diagnosis of leptospirosis.[15] Artefacts, such as fibrin threads in blood, are easily mistaken for leptospires. False positive diagnosis can frequently occur. DFM is therefore useful only to those with considerable experience in observing leptospires.[10]

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Phase Contrast Microscopy: Phase contrast microscopy is 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 DFM is available.[2]

Silver Deposition Techniques: The standard stains for spirochaetes have long been silver deposition methods. These depend on the ability of the method to selectively coat the surfaces of the leptospires with a deposit of silver. They require a large number of intact organisms to be present; artefacts are a major problem.[16] Smears made on slides can be stained with Fontana stain. Leptospires can be stained in formalin fixed tissue sections by the Dobell method for blocks, or Warthin-Starry, Faine, or Dieterle methods for cut sections. Well stained sections show black, thin spirochaetes on a pale yellow or colourless background.[17] PD-ADMAS modification yields superior results in terms of clear background of the stained slides and demonstration of the fine coils of the leptospires. The advantages of the modified technique are that over staining, clouding of the smear or development of artefacts or cracks of staining can be avoided.[12] Silver staining techniques are not specific for Leptospira and will also stain other spirochaetes and various other bacteria. It is tedious and difficult. It is not possible to determine the infecting Leptospira serovar based on silver staining results.[17]

Immunofluorescence: Immunofluorescence (IF) is useful in examination of urine, other body fluids, and tissues that have been frozen or are not amenable to silver staining.[17] IF is often preferable to silver staining because it is easier to see leptospires, especially in small numbers, and the serovars or serogroups can be determined presumptively. When a combination of antisera labelled with different fluorochromes is used, more than one serological type of leptospires can be identified in the same preparation. One disadvantage is the need for special fluorescent microscopy equipment; another is that specially prepared labelled antisera are required.[2] This is a fast and reliable test where facilities are available.[16] The antiserum used must be carefully evaluated for cross-reactivity with other bacteria and for specificity for the particular serovar (s) of interest.[17] IF has not been used widely for primary diagnostic tests.[16]

Immunohistochemistry: This can be achieved without the need for special equipment by using enzymatic or metallic labels on the secondary antibody. Phosphatase, peroxidase, or metallic gold-labelled antibody can be used in a variety of formats to stain leptospires in clinical specimens. This technique has the advantage of being useful with formalin-fixed tissue. Using modern methods of antigen retrieval in immunohistochemistry, leptospiral antigens can be detected and stored for considerable periods of time, making this technique particularly useful for retrospective studies.[17]

Nucleic Acid Probes and Hybridization: Leptospira specific sequences are isolated, cleaved and labelled with a reporter molecule. The labelled DNA in the single stranded (ss) form is then hybridized to ss DNA in the sample. If the nucleotide sequences in the nucleic acid probe are complementary to those in the sample, hybridization occurs and results in the form of nucleic acid hybridization which are monitored by autoradiography in the case of probes labelled with radioactive material, or calorimetrically with non-radioactive material.[2]

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Polymerase Chain Reaction (PCR): PCR can rapidly confirm the diagnosis in the early phase of the disease, when bacteria may be present and before antibody titres are at detectable levels. PCR can be applied to blood, urine, cerebrospinal fluid and tissue samples.[10] Ophthalmological complications of leptospirosis can be diagnosed directly and positively by PCR on aqueous humor.[17] PCR requires dedicated laboratory space and also highly skilled personnel. It may 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 if inhibitors are present in the clinical materials that are being examined.[10]

The main limitations of PCR-based methods are the need of special equipment, the relatively high cost of the reagents and the absence of automated and standardised procedures allowing the testing of large sets of samples[17] and the inability of most PCR assays to identify the infecting serovar.[3]

Culture of Leptospira: Leptospires can be recovered from blood or CSF obtained from patients during the septicaemic stage of illness or from urine during the immune stage. For routine use, Fletcher semisolid medium or Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid medium is recommended.[15]

Blood: Blood should be cultured in the first 10 days of the illness and before antibiotics are given. [10] Cultures are incubated at 28 to 30^0 C in the dark for 6 wks or longer.[15] All culture tubes are examined with DFM at 5 to 7 day intervals.[14] Blood culture is particularly valuable, as the serological response can be slow or even absent altogether if antibiotics are given early.[2] Isolated leptospires are identified either by serological methods or more recently by molecular techniques.[1] The sensitivity of blood cultures is low.[18] Therefore isolation and culture are primarily used for retrospective diagnosis.[19]

Urine: Leptospires appear in the urine from about 14^{th} day onwards and the same method of examination as for blood is used. Antibodies may also be present in urine and therefore it is essential to get the sample to the laboratory without delay as the leptospires will die even in alkaline urine.[6] Since urine is acidic and decreases the viability of leptospires, it should be inoculated into the medium within 2 hours after voiding. Viability is reported to be increased in urine samples neutralized with sodium bicarbonate and by using phosphate-buffered bovine serum albumin solution.

Cerebrospinal Fluid: Leptospires can be isolated by inoculating 0.5ml CSF into 5 ml of the semisolid culture medium during the first weeks of illness.[10]

Indirect Evidence:

Serological Methods: The wide range of tests that are available are broadly divided into genus-specific and serogroup/serotype-specific tests.[2]

Genus Specific Tests: These tests are based upon the use of a single antigen common for the genus *Leptospira*.[6] They become positive earlier in the illness and are ideal for a clinical diagnosis. The antigen for these tests is prepared from the non-pathogenic L. biflexa Patoc –1 strain.

Macroscopic Slide Agglutination Test: This test is carried out with a dense suspension of leptospires, which agglutinate into clumps visible to the naked eye.[2] They are performed on slides or

plates. Formalin killed antigens are mixed with serum to be tested and viewed with naked eye for presence or absence of agglutination.[6] It allows a provisional diagnosis of acute leptospirosis to be made within a few minutes; It has good correlation with both IgM ELISA and MAT, and therefore can be used as a valuable and simple screening test. The sensitivity of this test can be enhanced by adding the locally-prevalent serovars. It is a simple, rapid, and sensitive diagnostic test for active leptospirosis. It is however not suitable for retrospective testing. Positive reactions, however, should be confirmed by MAT.[2]

Indirect Fluorescent Antibody (IFA) Test: Specimens of blood, urine and parenchymatous organs are stained with luminescent sera and examined under a fluorescent microscope. The antigen antibody complex fluoresces brightly and is visible under the microscope. By this method living, dead or even fragmented leptospires can be demonstrated. This test is particularly helpful in the urinary shedders. The test is based on the ability of a specific serum treated with fluorochrome to combine with a specific antigen. This test carries a very high sensitivity and specificity.[6] When the IFA assay was compared with MAT, it was found that the IFA test is moderately sensitive and specific for the initial diagnosis of leptospirosis.[2] It is not dependent on the organisms being intact and lying in the plane of view nor does it require the organisms to be viable as does DFM or culture, but it requires a relatively expensive high quality incident light fluorescence microscope.[16]

Sensitized Erythrocyte Tests: Leptospiral extracts are used to sensitize sheep and human red blood cells.[2] Cells are mixed with sera containing homologous antibodies. These are sensitized erythrocytes agglutination [SEA] and haemolysing reaction [HL]. The haemolytic test is quite sensitive and adequate in human leptospirosis.[6] The same antigen–antibody system is involved in SEA and HL reactions. Compared to the CFT, the HL has the advantage that the complement used in the tests needs to be in excess and therefore it need not be titrated accurately before each test. But it has the disadvantage that the test sera need to be absorbed free from heterophile antibody.[2]

Complement Fixation Test [CFT]: CFT is performed using either whole leptospiral cells or soluble extracts. The CFT is useful in detecting relatively recent infection.[6] Complement fixation were widely used but methods were not standardised. Complement fixation tests have now been replaced by ELISA methods.[3]

Enzyme Linked Immuno Sorbent Assay (ELISA): It is a very sensitive and specific test for the biological diagnosis of leptospirosis. It is of particular value as a serological screening test because of its relative simplicity in comparison with the MAT. ELISA usually detects only the antibodies reacting with a broadly reactive genus-specific antigen and thus gives no indication of the causative serovar or serogroup. They can be performed with commercial kits or with antigen produced “in house”. A broadly reactive so called genus-specific antigen is generally used to detect IgM, and sometimes also IgG antibodies. The presence of IgM antibodies may indicate current or recent leptospirosis, but IgM-class antibodies may remain detectable for several years.[10]

The detection of specific IgM antibodies could be useful in particular in countries with a high degree of endemicity to help distinguish between acute or recent leptospirosis and past leptospirosis.[20] Stable reagents are available and form the basis of bedside tests, which are read visually. The use of computer assisted automated readers and the appropriate controls improves the

reproducibility and predictive value of this test.[2] ELISA methods have been applied in a number of modifications.[21] An IgM specific dot- ELISA was developed in which polyvalent leptospiral antigen was dotted onto nitrocellulose filter disks in microtitre tray wells. A commercial IgM dot ELISA dipstick has been shown to be as sensitive as a microtitre plate IgM ELISA.[3] Assays which detect IgM give positive results earlier in the acute phase of the disease.[22] Immunodot ELISA was comparable with Patoc agglutination for sensitivity, while a similar "line blot" immunoassay was comparable with Patoc agglutination and more sensitive than MAT. A further adaptation of IgM ELISA consists of a dipstick assay using a classical heat-extract of L. biflexa as antigen. Its main appeal is its ease of use, compatible with field conditions encountered where there are few medical resources. This type of test is essentially useful only for screening as it is limited by the same constraints that apply to conventional ELISA methods.[17] A single IgM positive sample taken during an acute febrile illness with symptoms suggestive of leptospirosis can provide presumptive evidence of infection.[23]

Some ELISA test systems are less specific than the MAT. Weak cross reactions due to the presence of other diseases may be observed. ELISA results should therefore be confirmed by the MAT. This may require testing a follow-up sample if the initial sample was taken at an early stage in the infection when the ELISA test may be positive, but the MAT negative. Where no antibody is detected or only a low titre is found, a second serum sample can be examined for seroconversion or a significant rise in titre.[10]

Microcapsule Agglutination Test (MCAT): This test is based on the passive agglutination of synthetic polymer carriers, sensitized with mixed antigens of sonicated leptospires, by leptospiral antibody. The one point MCAT kit was evaluated for use in humans by six WHO collaborating Centres for reference and research on leptospirosis. It is simple and can be performed by relatively unskilled personnel with minimum laboratory facilities; it is also very stable and can be kept for long periods without critical storage requirements. It gives positive results earlier in the course of the disease than MAT or IgM ELISA, but on the other hand it could not detect antibodies against some serovars, e.g. sejroe or the australis serogroup, and it may not detect antibodies in sera collected more than 1 to 2 months after the onset of disease.[2]

Lepto Dipstick: This dipstick assay for the detection of Leptospira-specific IgM antibodies in human sera uses heat stable antigen prepared from *Leptospira biflexa* and coated onto the lower band and internal control set up in the upper band which validates the performance of the assay, detection agent is also incorporated. The highly stable reagents and simple implementation makes this method suitable for use in clinical and field laboratories in tropical countries. The performance of the dipstick assay is useful for single serum specimens, it is recommended for use with paired serum samples, because besides strong staining, seroconversion or an increase in staining intensity are consistent with active leptospirosis. The sensitivity and specificity of the dipstick assay and the IgM ELISA agree well. The dipstick assay revealed cross reactivity with sera from patients with HIV, Hanta virus, 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 numerous practical advantages of the dipstick assay can contribute to an improved diagnosis.[2]

Latex Agglutination Test: This test depends on the sensitisation of commercially available latex particles with a leptospiral antigen. Antiserum will react with the antigen to cause agglutination of the particles. It provides a useful screening procedure.

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Lepto Dri-Dot: The Dri-Dot assay is a modified version of the latex agglutination assay using activated latex particles stabilized by freeze drying. The Dri-Dot assay has a relatively high sensitivity for samples collected at an early stage of the disease. The sensitivity of Dri-Dot assay is highest for samples collected more than 10 days after the onset of the disease. It gives a fast result by performing just a single reaction. No special equipment is needed. The cards can be stored at ambient temperature, with no need for a cold chain. Although performing the test requires some degree of skill, inter reader variability between skilled persons is low. But there is some risk of false negative results for samples collected early in the disease and also of false positive results.[24]

SEROGROUP/SEROVAR SPECIFIC TEST:

Microscopic Agglutination Test (MAT): MAT is the reference method for serological diagnosis of leptospirosis.[3] The MAT is a test, which determines agglutinating antibodies in the serum of a patient by mixing it in various dilutions with live/killed leptospires. Anti-leptospiral antibodies present in the serum cause the leptospires to stick together to form clumps. This clumping is observed using dark field microscopy. Agglutinating antibodies can be of both IgM and IgG classes. The MAT cannot differentiate between agglutinating antibodies due to current, recent or past infections.[10]

MAT is carried out with suspensions of living cultures or killed cultures. The use of a battery of strains giving comprehensive coverage of all serogroups–the multi-antigen MAT–provides an alternative to the so-called 'genus-specific' tests as a means of diagnosing leptospirosis; but the necessity to maintain large number of living strains of L. interrogans limits its use to reference laboratories. Wherever possible, local isolates of known identity should be included in the battery of strains because this has repeatedly been shown to increase both then sensitivity and the specificity of the test. A recent advance is the availability of standardized preparations of dried leptospires.

The degree of agglutination ranges from 100%, when no free leptospires can be seen between the clumps, through lesser degrees, as the serum is more diluted, to nil, as seen in the negative control suspension of leptospires in diluent. The degree of agglutination can only be assessed in terms of the proportion of free leptospires. The accepted endpoint of an agglutination reaction is the final dilution of serum at which 50% or more of the leptospires are agglutinated.

Interpretation of Diagnostic MAT: In a nonendemic area any level of antibodies, however low, may signify leptospirosis in the 1st week of a clinically compatible illness. The titre will rise in a second specimen taken after 3 to 7 days. If the titre remains below 100, even on repeated testing, it may be assumed that it was due to previous leptospirosis, and not due to current illness. A titre of 400 to 800 or more, or a 4-fold rise in titre between 2 tests, is diagnostic when combined with a clinical illness compatible with leptospirosis. In endemic areas, the diagnosis will be confirmed if the titre rises on retesting, but will be negated if it is unchanged, assuming that the infecting serovar was included among the antigens for the MAT.[2] In the current CDC case definition, a titre of > 200 is used to define a probable case with a clinically comparable illness. In areas where leptospirosis is endemic, a single titre of > 800 in symptomatic pts, is generally indicative of leptospirosis.[3]

The major advantage of the MAT is its high specificity.[10] The MAT is the serological test used in reference laboratories, because of its high degree of sensitivity and specificity.[25] MAT remains very useful for epidemiologic studies, identification of strains, assessment of the probable infecting serogroup and confirmation of illness for public health surveillance.[26] One important disadvantage is the need for facilities to culture and maintain panels of live leptospires. Furthermore, the test is both

technically demanding and time consuming, particularly when the panel is large. An obvious but definite shortcoming is that antibodies may not be detectable when the causative strain is not represented in the panel or only a low titre is found with a serovar that antigenically resembles the absent causative serovar.

The finding of no titre or a low titre in the MAT does not exclude leptospirosis in these circumstances. It is never possible to be sure that the panel is complete since new, unidentified leptospires may cause disease. The MAT cannot be standardized because live leptospires are used as antigens. Since test results may vary slightly from day to day, paired samples are best examined together. A degree of standardization can be achieved by using formalin-preserved leptospires as antigens. Unfortunately, preserved antigen denatures after only a few weeks. [10] One of the limitations of serodiagnosis by MAT is the prolonged period after recovery for which the agglutinating antibodies can be detected.[27] Application of the test also requires detailed knowledge of the locally occurring strains, as the predominant serovars have to be selected for use as antigens. The use of the MAT is thus restricted to specialized and well equipped laboratories.[28]

Serotype–Specific ELISA: Several attempts have been made to develop serotype specific ELISA tests with a variety of extracted antigens. Tests based on ultrasound-disintegrated or phenol extracted preparations show considerable serotype specificity.[2]

CONCLUSION: Numerous tests are available to diagnose leptospirosis. Depending on the situation whether diagnostic or epidemiological, appropriate test can be selected.

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