

## UPDATES IN LEPTOSPIROSIS DIAGNOSIS

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### ABSTRACT

Leptospirosis is an under-diagnosed disease due to its protean clinical manifestations and due to the non-availability of acceptable antigen based tests. The incidence of the disease depends on the distribution of leptospirologists. Culture takes time as the organism is slow growing. Microscopic agglutination test (MAT), ELISA for IgM and IgG antibodies, Polymerase chain reaction (PCR) are done. There is controversy regarding the use of darkfield microscopy (DFM). Earlier workers reported pseudospirochaetes in blood. It is also believed that leptospire are seen in low concentration in blood and disappear after one week. Introduction of darkfield stop has reduced the usefulness of DFM to visualize spirochaetes. But microbiologists working with DFM have not noted this fact. This review article gives an update on the usefulness of DFM, MAT, ELISA and PCR in the early diagnosis of leptospirosis. Standardization of DFM and PCR could help in assessing the real burden of leptospirosis in the world.

**Key words:** DFM, MAT, ELISA, PCR, pseudospirochaetes, darkfield stop

### INTRODUCTION:

**A**dolf Weil described leptospirosis as hemorrhagic jaundice in 1886. Stimson in 1907 observed slender spirochaetes in silver stained sections of kidneys from a fatal case of jaundice<sup>1</sup>. The causative agent of weil's disease was isolated by Inada in 1915 and named it as *L. icterohaemorrhagiae*<sup>2</sup>. This review article aims at giving updates in leptospirosis diagnosis which could help to find the actual magnitude of the leptospirosis problem in the world.

### SPIROCHAETES IN BLOOD

Helen Chambers found a new spirochaeta in the blood of Grave's disease patients and in normal individuals in 1913<sup>3</sup>. Hall in 1925 reported spirochaete-like filaments in dengue patients and normal individuals<sup>4</sup>. Vervoort in 1926 observed that

real leptospire could be differentiated from pseudospirochaetes by the experienced workers<sup>5</sup>. Wolff in 1954 observed that the differential centrifugation technique of Ruys could enhance the chances of demonstrating leptospira in the blood of patients. It involves collection of blood in either 1% liquid (sodium polyanethol sulfonate) in sterile saline or 1% sodium oxalate in phosphate buffer pH 8<sup>6</sup>. White and Ristic in 1959 could see leptospira in the urine of experimentally infected animals with *L. pomona* cultures by darkfield microscopy (DFM)<sup>7</sup>. Simpson in 1963 showed ultrastructural variations between hooked and nonhooked leptospire by electron microscopy<sup>8</sup>.

At the institute of microbiology, Madurai Medical College leptospire were seen using Carlzeiss darkfield microscope in 1968 in the blood of jaundice patients with renal complications and

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microscopic agglutination test (MAT) results were obtained from Indian Veterinary Research Institute, Uttarpradesh. Smith in 1975 found pseudospirochaetes in the blood of a fever case and a normal person using a binocular microscope with oil darkfield condenser and observed it can lead to erroneous diagnoses of leptospirosis<sup>9</sup>. For an ICMR project antigen from Nichol's strain of *T. pallidum* grown in rabbit testes and purified by differential centrifugation technique could be prepared in 1975 at the Central VD reference laboratory, Chennai. At first low speed of 3000 rpm was used to sediment testicular materials and next high speed of 10000 rpm to sediment treponemes<sup>10</sup>.

### DIAGNOSIS OF LEPTOSPIROSIS

It is believed that in nonendemic areas by the time leptospirosis is suspected, antibodies would have been produced<sup>11</sup>. Turner in 1973 observed that leptospire could be cleared from blood by immune defense mechanisms of the host in one week<sup>12</sup>. Everard in 1992 observed DFM is useful in examining cultures only<sup>13</sup>. WHO in 1999 recommended MAT and culture for the diagnosis of leptospirosis<sup>14</sup>.

### DARKFIELD MICROSCOPY

Since 1987, differential centrifugation technique was followed at the Institute of Microbiology, Madurai Medical College because of the vast experience with *T. pallidum* from 1975 to 1980. Carlzeiss darkfield microscope with special darkfield condenser was used. Leptospire could be seen in nonicteric, icteric and chronic cases of suspected leptospirosis. Some patients showed 50 leptospire per high power field (HPF) and with hooked ends also<sup>15</sup>. In 1998, Sugandhi rao et al found 27.27% (12/44) positivity for leptospira

by DFM in suspected leptospirosis cases with hepatorenal involvement<sup>16</sup>. Vijayachari et al in 2001 found 40.2% (37/92) sensitivity and specificity of 61.5% (48/78) for DFM using MAT and culture positive and negative cases<sup>17</sup>. Chandrasekaran and Gomathi reported DFM can be a standard screening test for the early and rapid diagnosis of leptospirosis as it correlated well with SERION ELISA kits for leptospira IgM antibody<sup>18</sup>. In 2012, Chandrasekaran and Ganesan reported 70% (68/97) sensitivity and 62.4% (156/250) specificity for DFM using ELISA IgM positive suspected leptospirosis cases and blood donors. Since leptospire could be found in low concentration in blood donors high speed centrifugation may not be necessary<sup>19</sup>. Ganesan et al in 2011 reported 80% (598/748) of MAT positive cases were DFM positive<sup>20</sup>.

In 2013, we were supplied with Olympus binocular microscope provided with darkfield stop and oil immersion objective fitted with darkfield stop to reduce the numerical aperture. It provided darkfield but Patoc cultures of leptospira did not show any leptospira. Darkfield Stop obstructs most of the direct light and illumination is reduced. After changing over to Magnus binocular microscope provided with oil darkfield condenser leptospire could be seen in Patoc cultures but brightness was not equivalent to Carlzeiss. Nowadays a single microscope is marketed to provide darkfield and phase contrast. People have mistaken the diagram of phase contrast microscope for darkfield<sup>21</sup>. Even textbooks like Prescott et al describe darkfield stop to help in seeing spirochaetes which is not proved technically<sup>22</sup>. It is time that every microbiology laboratory should have a conventional darkfield microscope and to do DFM on nonicteric, icteric and chronic cases of suspected leptospirosis.

## CULTURE OF LEPTOSPIRA

Earlier workers inoculated blood of patients and normal persons in broth media and found pseudospirochaetes. Later rabbit serum media (Stuart's, Korthoff's and Fletcher's) and EMJH (Ellinghausen, McCullough, Jhonson and Harris) medium were made available. Commercial EMJH medium is expensive. EMJH medium has been successfully prepared in a Medical Mission hospital laboratory in Kolenchery, Kerala. Based on increased leukocyte count, decreased platelet count, albumin in urine and increased urea level leptospirosis is suspected before giving antibiotics and blood in anticoagulant solution is collected and used for culture in EMJH medium but DFM is not done. Culture becomes positive in a few weeks and actively motile leptospire could be seen. Identification of cultures can be done by using antisera for MAT. To find out antibodies by MAT local serovars should be regularly subcultured. As it takes a few weeks for culture to become positive it cannot be used for early diagnosis of leptospirosis.

## SEROLOGY IN LEPTOSPIROSIS

Schuffner and Mochtar in 1926 described the phenomenon of agglutination and lysis with human and animal sera using live leptospire. Various factors like age of culture, density and cross reacting antigens between different serovars are to be taken into account while determining the serovar specificity. MAT requires maintenance of leptospiral serovars, use of good quality antigen and correct determination of endpoint<sup>23</sup>. In a reference laboratory in Australia about 5000 sera were tested every year from 1985 by MAT using a panel of 21 serovars. The common symptoms were headache, myalgia, sweats, chills, mild to severe fever, nausea, vomiting, renal involvement,

conjunctival suffusion, vision disturbance, respiratory distress and rash. 57% of patients were hospitalized. Duration of stay varied from 2 to 19 days. Serovars zanoni and pomona accounted for majority of hospitalizations<sup>24,25</sup>. In the Azores islands, 362 hospitalized patients (Sao Miguel-240 and Terceira-122) were tested by MAT from 1993 to 2000. Fever, chills, myalgia, headache and jaundice were the clinical symptoms. Fatal cases in young people were not laboratory confirmed. They were diagnosed clinically and progressed rapidly to acute renal failure and respiratory distress syndrome<sup>26</sup>. These results show that the disease is endemic in these countries and MAT may not be useful in detecting acute cases dying of leptospirosis.

Of the other serological tests ELISA has been thought of as a good alternative by Adler et al in 1980. IgM antibodies appear first and can be detected early in the disease. Later IgG antibodies are formed and persist for a long time<sup>27</sup>. ELISA detects IgM antibodies earlier than MAT<sup>28</sup>. Hence it detects current disease and useful to initiate specific antibiotic treatment. However, ELISA may be confirmed by MAT to rule out false positivity. Commercial ELISA kits can be evaluated by various workers in addition to DFM. MAT can be done in reference laboratories.

46 sera from acute suspected leptospirosis cases were sent from Madurai Medical College to a reference centre in Royal Tropical Institute, the Netherlands in 1994. MAT was negative in all sera but ELISA gave low titres in some sera. Later in 1996 sera samples from panuveitis cases were sent to the same Institute. Of 23 sera, 9 gave high titres with both MAT and ELISA. These results clearly show that serology is less sensitive in detecting

acute cases of leptospirosis. In 1998, DFM positivity for leptospira was found to be 82% (186/226) among panuveitis cases<sup>29</sup>. Rathinam Sivakumar of Aravind Eye hospital got MAT positivity in panuveitis cases through a reference laboratory in USA and proved leptospira can be an etiology of panuveitis for the first time in the world.

### MOLECULAR TECHNIQUES

Detection of leptospiral DNA by nucleic acid hybridization with <sup>32</sup>P and biotin labelled probes was described by Terpstra et al in 1986<sup>30</sup>. Eys GJJM Van et al in 1989 described PCR technique for the detection of leptospiral DNA in urine. 79 sera were tested by PCR using two primer sets G1/G2 and B64-I/B64-II. 75% of culture positive sera were positive by PCR. 60% of culture negative sera were also positive by PCR. The whole procedure takes 2 days. It is cumbersome and vulnerable to contamination<sup>31</sup>. There is a need to perform DFM along with standardized PCR technique.

### ANIMAL LEPTOSPIROSIS

As it is a zoonotic infection, detection of leptospirosis in animals and treating them with antibiotics can prevent urinary shedding of leptospores which can contaminate waterbodies used by man for bathing and drinking. Many leptospiral isolations could be achieved by Gangadhar and Rajasekar from animals and man<sup>32</sup>. Chandrasekaran and Pankajalakshmi found DFM to be useful in demonstrating leptospira in 2 out of 3 police dogs having fever and jaundice in 1997<sup>33</sup>. Ellis noted ELISA could be used to find leptospiral antibodies in animals.

### TREATMENT AND PROPHYLAXIS

Early detection of leptospirosis can lead to specific antibiotic treatment and prevention of later complications. Penicillin should be given intravenously 6 hourly for 7 days for severe cases requiring hospitalization. In addition, symptomatic treatment may have to be undertaken when there is renal complication. Amoxycillin, Erythromycin or Doxycycline can be given orally for 7 days in less severe cases<sup>34</sup>.

Prophylaxis involves close co-operation with Veterinarians to prevent urinary shedding of leptospores by infected animals. Waterbodies protected from animals should be used by man. Water tanks should be closed fully. Children should avoid playing in rain water contaminated with animal urine. Plasters should be used to cover wounds to avoid entry of leptospores in rain water or stagnant water. Waterbodies can be tested for the presence of leptospores. Pets can be immunized.

### CONCLUSION

DFM with a conventional darkfield microscope and ELISA kits for detecting IgM antibody to leptospira can be employed for early diagnosis of leptospirosis. MAT can be performed in a reference laboratory for identification of leptospiral serovars and to detect leptospiral antibodies. PCR has to be standardized and studied with DFM and culture of leptospores. This could reveal the actual burden of leptospirosis in the world.

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