

Technological Advances in the Diagnosis of Leptospirosis: Current Immunological and Molecular Tools

Linda Rose Jose¹, Dr. M N Sumana²

^{1,2}JSS Medical College, JSS University, Mysore, India

Abstract: *Leptospirosis is a worldwide zoonotic neglected infectious disease. It is common in tropical endemic areas including our nation. It is underreported and underdiagnosed in our country either due to the lack of awareness or due to the lack of proper diagnostic facilities. Leptospirosis should be considered during the differential diagnosis of other tropical febrile illnesses like dengue, malaria, rickettsial infections, hanta virus infections and many more. As leptospirosis in its severe form lead to multiorgan dysfunction and death, early and appropriate diagnosis and treatment is essential. This review concentrates on the diagnostic aspects of the neglected tropical disease.*

Keywords: Leptospirosis, Diagnosis

1. Introduction

Leptospirosis is a zoonotic disease of ubiquitous distribution, caused by spirochaetes of the genus *Leptospira*. In the areas of high prevalence, the disease results in high morbidity and considerable mortality [1]. Leptospirosis causes an extremely wide spectrum of human diseases that range from subclinical infection to multiorgan dysfunction which is often fatal [2]. It is more common in the tropics and subtropics where most developing countries are found. Hence China, South east Asia, South and Central America and Africa have many areas where the disease is endemic. The greater menace of leptospirosis in tropics and subtropics can be largely attributed to climatic and environmental conditions and also to the greater likelihood of contact with *Leptospira* contaminated environment [3]. The transfer of organisms to humans through contact with body fluids and urine of infected animals through mucosal surfaces or breached skin makes it a serious threat [1]. Due to its nonspecific symptoms that mimic better known diseases like dengue, malaria, influenza, meningitis, hepatitis or viral hemorrhagic fevers, leptospirosis has been frequently under diagnosed and under reported [3]. A few among these infections, in particular dengue, may give rise to large epidemics and the final outcome of which is that the cases of leptospirosis that occur during such epidemics may be overlooked [4].

In India, Kerala, Tamil Nadu and Andamans are endemic for leptospirosis. But now with better facilities to detect the disease, it is being reported from almost all parts of India [5]. In this review, we focus on the immunological and molecular diagnostic techniques available. In a study conducted in 2000-2001 by National Reference Center, Regional Medical Center (ICMR), Port Blair, a seropositivity ranging from 0 to 46.8% amongst all cases of fever was observed from various parts of India. The highest positivity rate was in South India at 25.6%. It was 8.3%, 3.5%, 3.1%, and 3.3% in northern, western, eastern and central India respectively [6, 7].

Diagnosis of leptospirosis in a laboratory is a challenge to all clinicians. There are several diagnostic techniques available for leptospirosis. According to the methodology used, it can be divided into a) methods demonstrating the organism in culture or clinical specimens, b) immunological methods and c) genomic studies [1].

2. Current Diagnostic Techniques Used

1. Microscopic demonstration:

In experienced hands, the isolation of leptospire is the most sensitive method of demonstrating their presence provided antibiotic residues are absent. Leptospire can be visualized in clinical material by dark field microscopy or by immunofluorescence or by light microscopy after appropriate staining [2]. This is the simplest procedure in demonstrating the organism. Dark ground microscopy of blood, urine, CSF and dialysate fluid has been done usually. A quantitative buffy coat method was recently shown to have a sensitivity of approximately 10^3 *Leptospira*/ml [2]. Microscopic examination of blood is of value only during the initial days of acute illness, when septicemic phase occurs. Also misinterpretation is a possibility as fibrin or protein threads can be misunderstood for leptospire as they may show Brownian movement [2].

The use of staining has increased the sensitivity of direct microscopic examination. Immunofluorescence and immunoperoxidase staining can be used for the staining of blood and urine. Histopathological stains like silver staining and Warthin Starry Stain are also widely used. Immunofluorescence microscopy is a technique that cannot be used in a routine setup. Immunohistochemical methods have been employed more recently [2].

2. Isolation of Leptospire:

Isolation of pathogenic leptospire is proof of an infection. Through isolation, serovar identification also becomes possible. This will aid in the recognition of new patterns of disease presentation, in disease surveillance and also in

assessing the intervention measures [4]. The samples should be collected for culture before the administration of antibiotics. Septicemic phase is the first stage of the disease and usually will be over by the end of the first week of acute illness. So, blood cultures should be taken as soon as possible after the patient's presentation. 2-3 drops of blood should be inoculated into 10ml of semisolid medium containing 5-Fluorouracil at the patient's bed side. To increase the recovery rate, multiple cultures should be done but this is rarely possible. CSF and dialysate can also be cultured during the first week of illness. From the beginning of the second week of illness, urine can be cultured. The excretion of leptospire in urine may last for several weeks. Survival of leptospire in urine is limited, so immediate processing is required. After centrifugation, the sediment is suspended in phosphate buffered saline and inoculated into media with 5-Fluorouracil. All the cultures are incubated at 28-30°C and examined weekly by dark field microscopy for about 13 weeks before discarding. Contaminated cultures may be passed through a 0.2µm and 0.45µm filter before subculturing to fresh medium [2].

3. Immunological Diagnostics

The majority of cases are diagnosed by serology. The antibodies become detectable in the blood within 5 to 7 days after onset of symptoms. Immunological tests can be divided into two: genus specific and serogroup specific. The genus specific tests include Complement Fixation Test, sensitized Erythrocyte lysis, macroscopic slide agglutination test, dot-ELISA, IgM dipstick assay, Latex agglutination test etc. [2].

The serogroup specific test which gives a definite serological investigation in leptospirosis remains the MAT. It is considered as the gold standard test in the identification of leptospirosis. MAT is the immunological reference standard for diagnosis of leptospirosis. But the test is cumbersome, in which reacting the patient's serum with different panels of live *Leptospira* antigens especially those that are locally common is performed. MAT detects both IgG and IgM. After incubation the mixture of serum and live leptospiral antigens are microscopically examined for agglutination and determination of titers are done. The test can be performed with live or formalin killed antigens. A single high titer or a fourfold rise in titer in the case of paired sera confirms the diagnosis. In the current CDC case definition, a titer ≥ 200 is used to define a probable case with a clinically compatible illness [8]. MAT is read using DGM.

A new approach to serological testing for leptospirosis by using *Leptospira* agglutination by Flow cytometry Light Scatter Analysis has been introduced [9]. In this test, the diagnosis of leptospirosis and the definition of the serogroup involved are feasible, based on the changes in the light scatter parameters like the forward scatter (FSC) and side scatter (SSC). The sizes and shapes of cells can be determined by measurement of FSC and SSC. FSC is related to the cell size and the optical refractive index of the outer membrane of the cell, SSC is related to the cell's granularity. FCM analysis was found to be objective, sensitive and rapid. The whole procedure will be taking around 1.5 hrs. The FCM analysis as a whole focuses on the monitoring of the

agglutination of various serovars following incubation with human serum [9].

Genomic Diagnostics:

Several diagnostic techniques can be employed in the genomic diagnosis. They are as follows:

PCR:

It involves the enzymatic amplification of target DNA sequences specific to the organism. Through a series of polymerizations which is carried out by heat stable DNA polymerase enzymes using primers which are short DNA fragments and they bind specifically to the sequence of interest. The amplified DNA produced by this reaction is visualized on agarose gel.

Several primer pairs were developed to diagnose all genospecies of *Leptospira* in which some primers were specific for genus *Leptospira*. And others were designed to identify only pathogenic species [10]. Some of the primers were based on species gene targets most frequently 16S or 23S RNA genes and repetitive elements [2]. Most of them were designed to amplify DNA from human samples. Several correlation studies have been carried out. Gravekamp *et al.* developed two groups of primers (G1&G2) and B64I and B64II) that were capable of diagnosing all genospecies of *Leptospira* known till 2003[11]. They do not amplify *L.kirshneri* serovar [2]. De Abreu Fonseca *et al.* [12] compared sensitivity and specificity of PCR against MAT and IgM ELISA in 124 serum samples. PCR was 100% specific but sensitivity was between 44 and 62% especially in those samples which was collected later on in the infection. In one study culture and PCR were positive in 48% and 62% of leptospirosis respectively but serology was positive in 87%.

Nucleic acid hybridization: dot blot

Nucleic acids are extracted by various ways from the samples and then these are immobilized directly on a solid surface (usually on a nitro cellulose membrane). *Leptospira* specific probes generated by molecular cloning technique or by purified total genomic DNA are then used to detect the pathogen nucleic acid [13]. It is a very specific technique that allows diagnosis of infection at a very early stage. The probe used should be specific. This will help in species differentiation. Several authors has applied dot blot to detect leptospirosis using either radiolabelled total genomic DNA or recombinant DNA probes [14, 15]. In a study by Pacciarini *et al.* (1993), the probe used could detect as little as 10pg of hardjo DNA (corresponding to 10^5 cells) [13].

Restriction endonuclease analysis:

The application of REA in *Leptospira* was first proposed by Marshall and colleagues [16]. It involves the extraction of double stranded DNA of *Leptospira*, digestion of DNA with restriction endonuclease followed by electrophoresis of digested DNA in agarose gel. This gives a specific DNA fingerprint. This pattern enables the identification of members of same species with same restriction sites. When the restriction enzymes cleave ds DNA at specific sequences most commonly 4 or 6 base pairs, a set of fragments will be generated. They migrate in agarose gel based on their

molecular weight. Thus a pattern of bands which is visible by UV light illumination is generated after staining with ethidium bromide. REA requires pure *Leptospira* culture and a trust worthy method for DNA extraction. Degraded or contaminated DNA will give abnormal and uninformative patterns. This method requires large amount of purified DNA which makes it unsuitable for identification of leptospires in body fluids. The advantage is that REA has enabled further genetic classification into subspecies or identify new *Leptospira* species [1].

Nucleic acid hybridization: Southern blot

Southern blot hybridization has been described as a valuable method for *Leptospira* classification by several authors [13]. It combines the REA and hybridization techniques. This provide information about polymorphic variations and relative homology between *Leptospira* serovars. The southern blot analysis gives smaller number of bands than REA and interpretation becomes easy. Southern blot hybridization provides information about the presence of particular sequences, the arrangement of sequences in the genome and homology with other strains. In study by Pacciarini *et al.* they had applied southern blot hybridization with specific recombinant probes for studies on the classification and homology of *L.interrogans* with clones containing repetitive sequences which will provide more informative patterns for serovar identification [13].

Amplified fragment length polymorphism/ Random amplified polymorphic DNA finger printing (RAPD):

The characterization into serovar is also possible by DNA "amplification finger printing or AFLP" [13, 1]. This assay involves the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. With appropriate annealing temperature, concentration of genomic DNA and cycle number, it is possible to obtain a characteristic spectrum of short DNA products, the number and size of which is specific for a particular strain or genetic variant. The disadvantage of this method is that it requires purified DNA and cannot be used for direct identification of *Leptospira* strains in biological samples [17, 18].

Pulse Field Gel electrophoresis:

In this technique larger genomic fragments are generalized with the help of restriction enzymes and is separated with gel electrophoresis. It is a cumbersome procedure but allows a relatively reproducible fractionation of an entire bacterial genome on a single gel [1, 2]. In a study using the enzyme Not I, most of the *Leptospira* serovars gave unique PFGE patterns [2].

DNA sequencing:

Sequencing nucleic acids at a particular genetic locus allows to identify interspecies differences and genetically classify different serovars. This is an expensive technique [1].

Ribotyping:

Ribosomal RNA (rRNA) is well conserved within the species. The phylogenetic position of bacteria can be identified by bacteriologists using probes on r RNA. With this technique a reasonably good correlation with the phylogenetic classification of *Leptospira* into 11

genomospecies has been demonstrated. A large database was constructed by using EcoR1 for digestion and 16S and 23SrRNA from *E. coli* as the probe [19]. Many serovars had unique profiles while some others could not be distinguished from from each other by ribotyping especially those that were previously known to be closely related such as icterohaemorrhagiae and copenhageni [20]. The accurate discrimination of serovar hardjo genotypes hardjobovis and hardjoprajitno has been possible with ribotyping [2]. The database is available at the Institute Pasteur website.(<http://www.pasteur.fr/recherche/Leptospira/Ribotyping.html>)

Matrix Assisted Laser Desorption Ionization –Time of Flight Mass Spectrometry:

MALDI-TOF MS is a rapid and easily applied method for bacterial classification at the species level. Mass spectrometry detects and compares individual protein mass peaks of bacterial cells. Either native bacterial cells (direct smear) or purified bacterial proteins can be spotted as samples. Some studies had mentioned the use of direct *Leptospira* samples such as blood and urine for spotting where as some studies emphasizes the use of extracted samples. MALDI-TOF MS mainly detects ribosomal proteins. Leptospiral strains are discriminated on the basis of protein peak patterns. Species level identification is possible with this technique. Species level identification is faster and reliable compared to other molecular typing methods. Rettinger *et al.* correlated the mass spectrometry analysis with the molecular sequence methods and proved that MALDI-TOF MS is reliable in determining *Leptospira* species [21].

Real Time PCR:

There are a limited number Real Time PCR assays available for detecting pathogenic *Leptospira* and the demerit is that none of them are clinically validated [22]. Few assays target genes were 16SrRNA (16S) and gyr B genes. A few targeted LipL32 and lig A and B genes which are restricted to pathogenic species. The real time PCR assays are commonly based on SYBR green technology and Taq Man probes and are more recently light upon extension technology [22].

A real time PCR assay could be a useful tool in rapid diagnosis of acute leptospirosis. It can be used especially in cases with rapid mortality before serology or culture is able to aid in the diagnosis. In a study by Stoddard *et al.* whole blood spiked with 10 leptospires/ml was culture positive only after 6 weeks of inoculation [22]. As real time PCR enables the quantitative monitoring of leptospiral cells there exists the opportunity to monitor treatment efficacy [23]. An extensive study has to be carried out to determine the performance of the assay on clinical specimens collected from patients at the different points during the course of infection. **Table:1** shows the merits and demerits of various available techniques.

Table 1: The merits and demerits of available diagnostic techniques

Techniques used	Merits	Demerits
Dark Field Microscopy	<ul style="list-style-type: none"> • Simple and easy • Useful when organisms are present in large numbers • Useful for observing agglutination in MAT 	<ul style="list-style-type: none"> • Takes time, practice and skill as artefacts such as fibrin threads in blood can often be mistaken as organism. • Not easy to see if concentration is low • Easier if alive – not so easy to identify if dead.
Culture	<ul style="list-style-type: none"> • Concrete evidence of the presence of live <i>Leptospira</i> • MAT and DNA analysis can both be performed on cultures 	<ul style="list-style-type: none"> • Exceedingly slow as leptospire take months to grow. • Often contamination problem occurs, therefore low sensitivity is an issue
Serology	<ul style="list-style-type: none"> • Screening for exposure to disease • MAT tells us about the serovar-epidemiologically useful • Less time consuming 	<ul style="list-style-type: none"> • Not as informative in acute stages of disease when the body is learning to recognise the antigen • Some human beings are “silent carriers” – no titres but they do carry <i>Leptospira</i> • Can’t always distinguish between vaccination and exposure titres.
Genomic diagnosis	<ul style="list-style-type: none"> • Rapid diagnosis • Monitoring of treatment efficacy possible 	<ul style="list-style-type: none"> • Expensive so mostly used for research purposes • Requires experience and versatility • Serovar identification not possible.

3. Conclusion

The development of an ideal diagnostic method for leptospirosis is a challenge. The assay should be less cumbersome, rapid, should have good sensitivity and specificity and also should be cost-effective. The gold standard for leptospirosis „MAT“ is cumbersome and greater chances of cross reaction exists. Immunological assays have shown promising results in early diagnosis but has several drawbacks. Genomic diagnostics is limited to research and genotypic analysis. Its establishment might offer an exciting possibility in early diagnosis of leptospirosis.

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5. Conflict of interests

The authors declare that they have no conflict of interest.

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