Present Status and Future Prospects in the Diagnosis of Leptospirosis

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Abstract

Leptospirosis is an occupational zoonosis affects 160 mammalian species. Timely and accurate diagnosis of acute leptospirosis is important for proper, prompt and early treatment in humans and animals. There are different approaches available for the diagnosis of leptospirosis. Currently, the disease is diagnosed by demonstration of leptospires, demonstration of antileptospiral antibodies, detection of nucleic acids of leptospires and animal experimentation techniques. This paper necessitates development of PCR based diagnostics to diagnose acute infections in all the species of animals and human beings using various clinical materials such as blood, urine, milk, semen, aborted materials and other tissue materials in future.

Key words: Leptospirosis – Diagnosis – Present status – Future prospects

Introduction

Leptospirosis is a spirochaetal disease and a major public health concern worldwide. The disease is considered to be the most widespread zoonosis in the world and affects 160 mammalian species. The disease causes enormous losses due to death of animals, decreased milk production, abortion, still birth and infertility. Humans become infected through the contact with the urine of infected animal. Rats and other rodents are the most important sources for human infection. Human infected with leptospirosis develop high fever, sudden onset of headache, nausea, vomiting, anorexia and muscle pain. Timely and accurate diagnosis of acute leptospirosis is important for proper, prompt and early treatment which potentially effective in mitigating the adverse consequences of leptospiral infection in humans and animals. There are different approaches available for the diagnosis of leptospirosis. Diagnosis can be made by demonstration of leptospires, demonstration of antileptospiral antibodies, detection of nucleic acids of leptospires and animal experimentation techniques. Hence this paper reviews the importance of different diagnostic techniques involved in the diagnosis of leptospirosis.

Conventional laboratory diagnostic methods

Traditionally the diagnosis of leptospirosis was depended upon Dark Field Microscopical examination, Giemsa staining technique, Silver staining method and Culture techniques for isolation of organisms.
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Dark Field Microscopical examination

Dark Field Microscopical examination is a simple and rapid procedure, the test has low sensitivity and specificity (61.5%) and (100.2%). Because the number of organisms present in the circulation is low during the acute stage of the disease. The organisms are excreted in the urine intermittently. Cellular fibrils, extrusions and fibrin strands mimic leptospires and may be mistaken for leptospires. Non-pathogenic leptospires could not be differentiated from pathogenic leptospires under DFM which lead to the test positive. Approximately $10^4$ organisms per ml are required to visualize one organism per field. Hence the test fails to detect active infections.

Staining technique

Various staining methods namely Giemsa, Silver impregnation etc., are used to stain the leptospires and seen under ordinary light microscopy. These techniques also have demerits similar to that of DFM. Hence these methods are not recommended for diagnosis of leptospirosis.

Culture technique

The leptospires are slow growing, fastidious organisms and require several days or weeks to grow and weeks to months for identification. Isolation of leptospires depends on the number of viable organisms in clinical samples. The tissue autolysis and over growth of contaminants hinders the isolation of leptospires. Use of selective agents may reduce the chances of isolation and many strains of leptospires will not grow in selective media containing multiple antibiotics. Isolated leptospires require either serological or molecular methods for identification. Hence rapid diagnosis which helps for the treatment of patients or animals is not possible by isolation of leptospires, even though it is foolproof of infection.

Leptospiral diagnostics - Present status

Due to the shortcomings of the above conventional diagnostic methods, Microscopic Agglutination Test, different formats of Enzyme Linked Immuno Sorbant Assay, Recombinant antigen based diagnostics and Polymerase Chain Reaction based diagnostics are being employed in different laboratories to diagnose leptospirosis.

Microscopic Agglutination Test

MAT is the gold standard test recommended by OIE (2008) and widely accepted test for diagnosis, screening of animals for international trade and epidemiological investigations (WHO, 1986). It is widely used for detection of leptospiral antibodies in sera using a battery of live antigens, for measuring antibody titre and for identifying unknown leptospiral isolates. Most of the studies on leptospirosis conducted in India as well as in other countries are based on MAT, because of its high sensitivity (96.6 per cent) and specificity (94.8 percent). The test detects leptospiral agglutinating specific antibodies from both recent and past infections. It is useful for epidemiological purpose. Since the animal once infected stays MAT positive for several years. The test requires a battery of antigens covering a range of serovars that are expected or likely to be circulating in a particular geographical area, where the patient becomes infected. Locally isolated strains are required to include in the panel which may give more specific and sensitive result than reference strains. At least one strains of saprophytic serovar (Patoc I) should be included in the panel which act as genus specific antigen to detect infections caused by emerging serovars in a particular geographical area.

The reciprocal of the highest dilution which shows 50 per cent reduction in the number of free leptospires comparable to the respective antigen control with or without agglutination was recorded as the end point titre and the test positive. One of the important issues of MAT is the cut off titre for diagnosis. Generally single titre of 1:100 or more are considered as positive. But based on the single titre of single serum sample, it is very difficult to prove current infection. The antibody titre might be due to residual antibody of past infection. A fourfold rise in titres between acute and convalescent sera is widely accepted as indicative of infection. So a second serum sample collected during the convalescent stage of the disease is required for demonstrating a raising titre which has a diagnostic significance. Sometimes confirmed patients may have low MAT titres and MAT is insensitive particularly in early active phase specimens. Moreover, patients with fulminant leptospirosis may die before seroconversion occurs. Cross reactive antibodies in syphilis, relapsing fever, lyme disease, enteric fever, dengue and malaria may also give a titre of 80 or 100. Obtaining second serum sample for demonstration of rising antibody titre is often difficult in routine practice. The patient or animal need immediate treatment and cannot wait for the result of second serum sample, which will be available at least after 14 days of 1st serum sample collection. Convalescent titres can remain as high as 1:800 for 13 months and 1: 192 for seven years after infection. Such persistence of antibodies together with antibiotics may lessen the immune response. Thus some individuals may become seronegative. Hence different laboratories use different cut off titres ranging from 100 to 400 for diagnosis based on endemicity or base line titre in the population. But the base line titre may vary from time to time, place to place and species to species. Hence it is not possible to have a single base line for all species of animals in entire country for years together. In these circumstances, the diagnosis may be made by correlating the MAT result with the result of dark field microscopical examination and clinical manifestations.

**ELISA**

ELISA is one of the techniques commonly used for the diagnosis. It is highly sensitive method. It detects IgM antibodies earlier than MAT. This test can be utilized to detect IgG and IgM antibodies separately in infected individuals. Large number of samples can be analyzed. The infecting serovar cannot be assessed, since single genus specific antigen is used.

**IgM ELISA**

This assay is based on the binding of *Leptospira* specific IgM antibodies to the *Leptospira* antigen. Bound IgM antibodies are detected in ELISA. The antibodies formed are predominantly of the IgM class during the acute phase of leptospirosis. Therefore the demonstration of specific IgM can help in the rapid diagnosis of the disease. This is particularly important in areas where leptospirosis is widespread and residual antibodies of past infections are found in a large part of the population. This test is considered to be 98% - 100% sensitive and 95 – 98% specific. IgM ELISA is useful for screening leptospirosis towards the end of the first week of illness. The ELISA’s for *Leptospira* IgM antibody by Serion – Verion (Chaudhry et al., 2006) and Panbio-diagnostics (Chourasia et al., 2006) are being used for the diagnosis of acute leptospiral infections and are available in the market. However the limited shelf-life of reagents and the requirements for an ELISA reader to read the results and a continuous electrical supply to keep the test materials in refrigeration limit its usefulness in developing countries.

**Leptospira dipstick assay**

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The lepto dipstick assay was developed by the Royal Tropical Institute (KIT), the Netherlands. The Dipstick method gave sensitivity and specificity of 77.57% and 91.3% respectively. Sehgal et al. (1999) also reported the sensitivity and specificity of 78.7% and 88.3% respectively. This method was used to detect IgM for diagnosis of the first stage of illness in the first week until one month. This IgM assay requires no electricity or refrigeration and is easier to use and less expensive. Considering the easy of storage and use high sensitivity and specificity and broad reactivity, the lepto dipstick may prove a valuable diagnostic tool, especially where laboratory resources are limited. But it requires 3 h incubation before the results can be read.

Lateral flow Assay

The Lateral Flow is a simple assay for the detection of Leptospira-specific antibodies in human sera and whole blood. The assay requires no special equipment and results are obtained within 10 minutes. The ingredients can be stored at +20°C to +25°C. The overall sensitivity (85.80%) and specificity (93.60%) of the Lateral Flow compares well with those of the IgM ELISA routinely used in the sero diagnosis of leptospirosis. The broadly reactive antigen allows the detection of Leptospira infections caused by a wide range of strains of different serovars. The Lateral flow assay for the detection of leptospira specific IgM antibodies is also available as Lepto check (Zephyr Biomedicals, India) in market.

Recombinant DNA based assays

Several diagnostic tests namely ELISA, Dipstick ELISA, LAT and Lateral flow assay have already been developed based on whole cell leptospiral antigen preparations to screen the leptospiral infection. The antigens used in these assays have been prepared using a variety of techniques, such as a hot phenol water extraction, sonicated whole cell leptospires, mechanical disruption, detergent extraction, acetic acid extraction, ethanol extraction, or SDS extraction. Variations in the techniques used for preparation of antigens have contributed to the poor reproducibility of the results. Recently recombinant antigens have been produced using porin Transmembrane protein ((Omp L1), lipoproteins (LipL32, LipL36 and Lip L41, LipL21 and a heat shock protein (HSP 58). Recombinant antigen based serological tests may achieve higher sensitivity than other tests because of the purity of the immunodominant antigen and the lack of non-specific moieties present in whole cell preparation. All these proteins have been used in ELISA for sero diagnosis of leptospirosis. These purified recombinant proteins are also easy to prepare compared to leptospiral LPS. The high degree of sequence conservation of these leptospiral proteins is a potential sero diagnostic advantage compared to the more variable LPS antigens.

When various recombinant protein antigens were examined by ELISA for their utility in serodiagnosis of leptospirosis, Lip L32 had the highest sensitivity and specificity. Hence recombinant multiepitope proteins containing LipL32, LipL21, OmpL1 and Loa 22 fusion proteins based ELISA have been developed and were found to have high accuracy than MAT (Lin et al., 2008 and Chalayan et al., 2011). But the sensitivity and specificity of these recombinant protein antigens have not been determined in vaccinated animals. Hence they cannot differentiate infected and vaccinated animals. Identification of leptospiral antigens expressed only during infection could be useful for the development of new diagnostics that might differentiate vaccinated and infected animals. Palaniappan et al. (2002 and 2004) identified leptospiral immunoglobulin like proteins, Lig A and Lig B which are only expressed in vivo. Purified recombinant proteins Lig A and Lig B were evaluated for their diagnostic potential in kinetic
ELISA using sera from vaccinated and MAT positive dogs. Sera from vaccinated dogs showed reactivity to whole cell antigens of leptospires, but did not show reactivity in the kinetic ELISA with recombinant antigens suggesting a lack of antibodies to Lig proteins in the vaccinated animals (Palaniappan et al., 2004). LigA based ELISA has been developed and used for early diagnosis of leptospirosis (Srimanote et al., 2008).

**Polymerase Chain Reaction**

Polymerase chain reaction is a rapid and specific test which can be applied in the molecular diagnosis of acute leptospirosis. Different workers have reported the application of PCR for the diagnosis of leptospirosis. Leptospiral DNA has been amplified from serum, urine, aqueous humor, cerebrospinal fluid and a number of tissues obtained at autopsy. It also used to detect leptospires in bovine semen, aborted fetuses and bovine mastitis milk. Using real time PCR, it is possible to quantify the amount of template and therefore the number of target organisms. Smythe et al. (2002) developed a real time Taqman PCR assay to detect leptospires in clinical and environmental samples. A real time SYBR green PCR assay targeting the Lip L32 virulence factor gene is currently being validated (Levett, 2004). Now a days, light cycler PCR technology has been used for rapid detection of pathogenic leptospires (Woo et al., 1998). Diagnosis of leptospirosis in human clinical samples using light cycler PCR technology has also been reported (Merien et al., 2005). A taqman assay was modified to suit the light cycler instrument and was found to have an analytical sensitivity of 10 copies per reaction (Slack et al., 2007).

**Future prospects**

As the clinical signs of leptospirosis often are nonspecific, the disease is easily mistaken for other major infectious diseases. Thus laboratory testing to confirm the clinical diagnosis is essential for optimal treatment and patient management. Currently there is no sensitive, specific, low cost, rapid and widely available diagnostic test for leptospirosis. However the disease is diagnosed by dark field microscopical examination, MAT, Culture methods, ELISA’s and PCR. These existing methodologies suffer from several pitfalls to detect acute infections. The outer membrane proteins like Omp L1, Lip L41, and Lip L32 and Lig A and Lig B were found to be conserved only in pathogenic leptospires and are antigenic in nature. Using these proteins, with the advent of recombinant DNA technology, recombinant protein based immunodiagnostics have been developed by many workers which achieve high sensitivity and specificity. But these recombinant protein based assays have not yet been evaluated under field conditions. PCR also has the potential to make a dramatic impact in diagnosing leptospirosis. Numerous studies document its high specificity and sensitivity with the ability to identify early infection. In this situation, evaluation of assays utilizing recombinant antigens such as Lip L32 and Lig A and Lig B under field condition using large number of serum samples is mandatory. Development of PCR based diagnostics to diagnose acute infections in all the species of animals and human beings using various clinical materials such as blood, urine, milk, semen, aborted materials and other tissue materials will be a boon in the diagnosis of leptospirosis in future. Until that it is advisable to use a combination of PCR and MAT for the diagnosis of leptospirosis (Balakrishnan et al., 2009) instead of using either alone.

**References**
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