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## Preeti

Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar Haryana, India

## Laxmi Bai

Department of Veterinary Medicine, College of Veterinary Sciences Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar Haryana, India

## Ramkaran

Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar Haryana, India

## Ravi Kumar

Department of Livestock Production Management, College of Veterinary Sciences Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar Haryana, India

## Correspondence

### Preeti

Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar Haryana, India

## A review of laboratory tests: Leptospirosis diagnosis

Preeti, Laxmi Bai, Ramkaran and Ravi Kumar

### Abstract

Leptospirosis caused by pathogenic spirochaetes of the genus *Leptospira* is a zoonosis of global distribution. Humans become infected through contact with water or soil, contaminated by the urine of infected animals such as rodents, dogs, cattle and pigs. Either organism's isolation from the patient or on sero-conversion or a rise in antibody titre in the MAT is the definite diagnostic method. Some other tests used for diagnosis are- Dark field microscopy (DFM), Immunofluorescence (IF), Immunohistochemistry, Polymerase Chain Reaction (PCR), Enzyme Linked Sorbent Assay (ELISA), Indirect Haemagglutination Assay (IHA) and Lepto-Dipstick. Microscopic agglutination test (MAT) or culture has to be done to identify the specific serovar. The various available tests for leptospirosis diagnosis have been explained in this article.

**Keywords:** *Leptospira*, leptospirosis, weil's disease, dark field microscopy, microscopic agglutination test (MAT)

### Introduction

Leptospirosis is caused by pathogenic bacteria called leptospire that are transmitted directly or indirectly from animals to humans and considered as the most widespread zoonosis in the world (Musso and Scola, 2013; Vihol *et al.*, 2017) <sup>[1, 2]</sup>. It is a potentially serious but treatable disease. The disease leptospirosis is described as an occupationally transmitted disease. The risk of infection depends upon amount of exposure. Some people have a high risk of exposure owing to the environment in which they live. Humans contract leptospirosis through contaminated urine of chronically infected animal domestic or agricultural rodents, dogs, pigs and cattle (Rebecca and Deborrah, 2000) <sup>[3]</sup>. According to the WHO, the main occupational groups at risk of leptospirosis include agricultural workers, pet shop workers, veterinarians, sewer workers, abattoir workers, meat handlers, military personnel, survivors of natural disasters. Environmental conditions are an important influence on the incidence of leptospirosis; the disease is rare in deserts, common in warm, humid, tropical areas and seasonal rains and severe weather are associated with increased frequency of disease. Leptospirosis is found in a wide variety of environmental contexts, in industrialized and developing countries, and in urban and rural contexts (Everard and Everard, 1993) <sup>[4]</sup>. In India, outbreaks have been reported related to heavy rainfall in various parts of the country. In South-India, suspected cases are reported between June and October due to heavy rains and floods. Leptospirosis has been consistently reported from the Andaman and Nicobar group of Islands (thus called 'Andaman Haemorrhagic Fever') West Bengal, Kerala and Coastal Karnataka, India (John, 1996; Roy *et al.*, 2005) <sup>[5, 6]</sup>. Due to clinical presentation of leptospirosis is unspecific, misdiagnosis is frequent and diagnosis is based upon laboratory results.

### Historical perspective

In 1886, Adolf Weil described a clinical syndrome characterized by splenomegaly, jaundice, haemorrhages and nephritis (Weil, 1886) <sup>[7]</sup>. Leptospire were first identified as the cause of Weil's disease in Japan, where it was common among coal miners (Faine *et al.*, 1999) <sup>[8]</sup>. Clinical syndromes resembling Weil's description of haemorrhagic jaundice have been known for many centuries. Such diseases were recognized as occupational hazards of rice farmers in ancient China (Faine, 1994) <sup>[9]</sup>.

### Classification of *Leptospira*

The genera *Leptospira*, *Leptonema* and *Turneria* belong to the family of *Leptospirosis*. The families *Leptospirosis* and *Spirochaetaceae* (genera *Spirochaeta*, *Cristispira*, *Borrelia* and *Treponema*) make up the order *Spirochaetales*.

The genus leptospirais subdivided into two species, namely, the *L. interrogans* (pathogenic) and the *L. biflexa* (non-pathogenic). In routine for the speciation growth at 13 °C and in the presence of 8- azaguanine is being used (Johnson and Harris, 1967; Johnson and Rogers, 1964) <sup>[10, 11]</sup>. Both the species have several serovars and serovar is the basic taxon, which is defined on the basis of surface antigenic makeup (Brenner *et al.*, 1999; Kmety and Dikken, 1993) <sup>[12, 13, 14]</sup>. More than 250 serovars arranged into 25 serogroups have been described under the species *L. interrogans* (Galloway and Levett, 2010) <sup>[15]</sup>. The species *L. biflexa* has 65 serovars arranged in 38 serogroups.

### Epidemiology

Leptospirosis is an emerging global public health disease and most widespread zoonosis in the world (Vijayachari, 2008) <sup>[16]</sup>. Higher incidence of the disease occurs in warm than in temperate regions in the world. The number of severe human cases worldwide is estimated above 500,000 (WHO) <sup>[17]</sup>. Numerous animals, primarily mammals, are sources of human infection. Rodents are the most important and widely distributed reservoirs of leptospirais. Some serovars are associated with a particular species of natural maintenance host. In chronic infections, leptospirais 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 inter-human 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.

The endemicity of the disease is mainly located in the Caribbean, Central and South America, Southeast Asia and Oceania eco-epidemiological settings in which leptospiral transmission occurs can be broadly categorized as urban, rural, recreation associated and disaster sequel (Pappas *et al.*, 2008) <sup>[18]</sup>.

Occurrence of leptospirosis in cattle in India was documented by Adinarayanan *et al.* (1960) <sup>[19]</sup>. Subsequently, the prevalence of the disease in animals from various parts of the country has been reported by Rajasekhar and Nanjiah (1971) <sup>[20]</sup>, Srivastava *et al.* (1983, 1991) <sup>[21]</sup>, Varma *et al.* (2001) <sup>[22]</sup>, Piramanayagan *et al.* (2002) <sup>[23]</sup> and Sivaseelan *et al.* (2003) <sup>[24]</sup>. According to a NICD report (1997) <sup>[25]</sup>, prevalence rate of Leptospirosis is high in Andaman, Bengal, Gujarat, Karnataka, Andhra Pradesh, Tamil Nadu and Kerala. The incidence rate of leptospirosis in the Andaman Islands was estimated to be between 50-65 cases/100,000 per year, which is believed to be the highest in India (Sehgal, 1998) <sup>[26]</sup>.

### Clinical presentation

The clinically disease may range from a flu-like illness to a serious and sometimes fatal disease and may confused with other diseases, especially dengue fever and other hemorrhagic fevers. The mean incubation time is 1-2 weeks, with a range of 2 days to 30 days. The 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. In addition to hepatic or renal dysfunction, leptospirosis should be seriously considered in patients with pulmonary symptoms and fever (Edwards and Domm, 1960) <sup>[27]</sup>.

### Laboratory diagnosis

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 (Edwards and Domm, 1960) <sup>[27]</sup>. 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 leptospirais, by culturing them or by demonstrating an appreciable antibody response to them (Levett, 2001) <sup>[28]</sup>.

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 (Speelman, 2005) <sup>[29]</sup>. During the first week of illness diagnostic tests consist of blood culture, examination of sera collected in the first and third week for evidence of rising titre and culture of urine after the third week of illness (Sehgal, 1991) <sup>[30]</sup>.

### Direct Methods

**Dark-field microscopy:** During the first few days of the acute illness microscopy of blood is valuable. Dark field microscopy (DFM) examination of body fluids such as blood, urine and CSF has been used. By DFM for the possibility of visibility approx. 104 leptospirais/ml are necessary for one cell per field. If there are few bacteria in the sample, then this method not showing the positive value. Double centrifugation of the sample at low speed to separate the cellular elements, and then at high speed, help concentrate the leptospirais. It is the procedure of choice for the demonstration of the organisms in tissue fluids (Gangadhar and Rajashekhar, 1998) <sup>[31]</sup>. 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 (Chandrasekaran and Gomathi, 2004; Sonnenwirth, 1980) <sup>[32, 33]</sup>.

**Phase contrast microscopy:** Phase contrast microscopy is useful for visualizing leptospirais 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 (Sambasiva *et al.*, 2003) <sup>[34]</sup>.

**Silver deposition techniques:** The silver deposition methods are considered as the standard method of staining for spirochaetes (Ellis, 1986) <sup>[35]</sup>. Leptospirais surfaces are selectively coated with a deposit of silver. They require a large number of intact organisms to be present; artefacts are a major problem. (Faine *et al.*, 1999) <sup>[36]</sup>.

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 (Ellis *et al.*, 1982) <sup>[37]</sup>.

**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 (Ellis *et al.*, 1982) [37]. It is often preferable to silver staining because it is easier to see leptospire, 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 leptospire 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 (Levett, 2001) [28]. This is a fast and reliable test where facilities are available (Faine *et al.*, 1999) [36]. The antiserum used must be carefully evaluated for cross-reactivity with other bacteria and for specificity for the particular serovar (s) of interest (Ellis *et al.*, 1982) [37].

**Immunohistochemistry:** Enzymatic or metallic labels are used on the secondary antibody for this test. Phosphatase, peroxidase, or metallic gold-labelled antibody can be used in a variety of formats to stain leptospire 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 (Ellis *et al.*, 1982) [37].

**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 (Levett, 2001) [28].

**Polymerase chain reaction (PCR):** Direct Polymerase Chain Reaction (PCR) on specimens enables rapid and direct diagnosis, at least in the early and convalescent stages of infection. The reaction detects leptospiral DNA in the specimen, down to extremely small amounts equivalent to the DNA content of about 10 leptospire or less. A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar (WHO, 2003) [38]. It 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 (Bal *et al.*, 1994) [39]. Ophthalmological complications of leptospirosis can be diagnosed directly and positively by PCR on aqueous humor (Ellis *et al.*, 1982) [37]. 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 (Ellis *et al.*, 1982) [37].

Limitations - 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 (Ellis *et al.*, 1982) [37].

**Culture of leptospira:** Leptospire 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 (Johnson and Harris, 1967) [10].

#### Serological and other indirect methods

Most cases of leptospirosis are diagnosed by serology. Antibodies can become detectable by the 6th to 10th day of disease and reach peak levels within three to four weeks. Antibody levels may then gradually decline but remain detectable for years.

#### Microscopic agglutination test (MAT) (Galton *et al.*, 1958) [40]

The MAT is a sensitive assay, but because of the antigenic heterogeneity of *Leptospira* spp. requires a large number of serovars as antigens. In addition, it would not be useful at the early stages of the disease when the antibody to *Leptospira* spp. is not present or, if present, is at a low level in the CSF. Positive results are defined as a 4-fold rise in titer between acute and convalescent specimens. A single titer exceeding 1:200 or serial titers exceeding 1:100 suggest leptospirosis, but neither is diagnostic. Some patients have serological evidence of previous infection with a different leptospiral sero group. In these cases, serological diagnosis is complicated further by the "anamnesic response", in which the first rise in antibody titre is usually directed against the infecting serovar from the previous exposure.

#### Enzyme linked immunosorbent assay (ELISA)

This test relies on the detection of IgM antibodies which appear in the blood a day or so earlier than those used in MAT. There is often poor correlation between MAT and ELISA results on sera of individuals. The reference standard is MAT, IgM antibodies become detectable during the first week of illness, allowing the diagnosis to be confirmed and treatment initiated while it is likely to be most effective though, antibody levels are generally low or absent during very early infection (Terpstra *et al.*, 1985; Terpstra *et al.*, 1980) [41, 42].

Many studies have demonstrated Pan Bio ELISA to be more sensitive than MAT for detection of cases early in acute illness (Levett *et al.*, 2001) [43]. IgM antibodies start appearing during the first week of illness though antibody levels are low or not detectable very early on in the illness. Leptospirosis can be diagnosed on the basis of the presence of IgM antibodies by Pan Bio ELISA, in a single serum sample collected during the acute phase of the illness. A convalescent sample taken after two weeks is required to confirm the results. A limitation of using a single serum sample in the demonstration of IgM antibodies is the absence of antibodies very early on in the infection or the persistence of antibodies. IgM antibodies in leptospirosis persist for a long period with varying rates of decline (Ahmed *et al.*, 2005) [44].

The bacterial concentration is less in serum than fresh blood. Studies comparing the PCR and IgM have demonstrated PCR alone to be less sensitive than serological tests over the course of the disease; it was the most sensitive method in those samples with no demonstrable antibodies collected during the very early stages of the disease (Ooteman *et al.*, 2005; Fonseca and Teixeira de Freitas, 2006) [45, 46]. Therefore use of PCR in combination with IgM ELISA would improve the

sensitivity of the diagnosis of leptospirosis in the first phase of the disease.

#### Indirect haemagglutination assay (IHA)

IHA testing is a rapid and easily performed method of diagnosis that is based on genus-specific antibodies. However, contrasting results have been obtained through various studies done to find the sensitivity and specificity of IHA in early infections. It has been shown to have a sensitivity of 92% and specificity of 95% compared with MAT. It can be concluded that IHA has a very limited scope in diagnosing *Leptospira* infections before 8 days (Imamura *et al.*, 1974)<sup>[47]</sup>.

#### Leptodipstick assay

This is an assay that detects *Leptospira*-specific IgM antibodies in human sera (Gussenhoven, *et al.*, 1997)<sup>[48]</sup>.

**Conclusion:** Numerous tests are available to diagnose leptospirosis. Depending on the situation whether diagnostic or epidemiological, appropriate test can be selected. When using a single sample collected during the early, acute phase of the disease, results of Pan Bio IgM ELISA can give us a presumptive diagnosis of leptospirosis. Very early on in the infection it may even fail to detect the presence of antibodies. PCR is a sensitive and specific technique which can detect the presence of DNA in the very early stage of the disease, so PCR together with IgM ELISA can be used to confirm the diagnosis, early on in the acute stage of the infection. The bacteria are adapted to the environment of the tropical region with plenty of rainfall and it is often difficult to avoid exposure of the people to animals or contaminated environment. Because of this, early case detection and prompt treatment and creating awareness about the disease among the people and public health professionals are the steps that could be taken to reduce the magnitude of the problem.

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