Foreword

Leptospirosis is an important public health problem of India. Recently there has been frequent resurgence of this malady coinciding with the natural calamity. In spite of considerable advances in the management of this condition, timely and accurate diagnosis is often difficult, owing to its atypical presentation and similarity of signs and symptoms with other infectious diseases.

It is indeed a matter of great satisfaction to learn that RMRC, Port Blair in collaboration with WHO is bringing out the much needed manual on laboratory diagnosis of leptospirosis. This manual is a crystallization of more than a decade of experiences and endeavour of the scientists of RMRC Port Blair working in the field of leptospirosis. This is a meticulously planned manual presented in a simple way for its use in the laboratory for the diagnosis and characterization of leptospirosis. This book will be of significant utility and will prove to be a ready reckoner for the clinicians and the laboratory personnel as well.

I sincerely compliment the joint efforts of RMRC, Port Blair and WHO in bringing out this manual which I am sure will serve as a useful guide for early detection of the disease, and thus reducing the morbidity and mortality associated with leptospirosis.

(N.K. Ganguly)
Director General
Preface

Leptospirosis is becoming an increasingly significant public health problem, particularly in tropical developing countries. The whole of Southeast and South Asia are endemic to the disease. Frequent outbreaks are occurring, many of which in the aftermath of natural disasters. Yearly upsurges and outbreaks are common in rice cultivating regions as a large number of farmers get exposed to contaminated wet environment. Complications such as severe pulmonary haemorrhages and renal failure are being reported more frequently. Once these complications set in, it is difficult to save the patient even in most well-equipped hospitals and the case fatality ratio becomes very high.

In most of the developing countries where leptospirosis is endemic, no specific control programme is in operation and the surveillance is often incomplete. Therefore, the disease outbreaks continue unchecked and even an estimate of the disease burden is missing. Being a zoonotic disease with a large spectrum of animal carriers and the difficulty in preventing exposure of the people, whose subsistence depends upon small scale farming and other occupations closely linked to the environment, it is difficult to devise an effective control strategy. In this situation, early case detection and treatment becomes very important for reducing the morbidity and mortality.

The two obstacles for early case detection are the lack of awareness of the people and medical professionals about the disease and the unavailability of laboratory support for diagnosis. Because of the frequent occurrence of the disease either in the form of outbreaks or as sporadic cases, awareness, at least among the medical professionals is increasing. However, lack of laboratory support and trained laboratory manpower is still an important issue in leptospirosis surveillance and control. Several rapid test kits has become available in the market in the recent years. However, there is no uniform standard or algorithm for laboratory diagnosis. There is a need to systematically evaluate these commercially available tests and evolve a diagnostic algorithm.

This Centre has been carrying out research on leptospirosis for about one and a half decades now. Since 1999 it is working as the National Leptospirosis Reference Centre and since 2003 as WHO Collaborating Centre for Diagnosis, Research, Reference and Training in Leptospirosis. As part of these activities, the Centre has also been conducting Hands-on-Training Workshop on laboratory diagnosis of leptospirosis on alternate years.

We at this Centre thought it worthwhile to bring out a document based on the information generated by the research activities of the Centre and the Collaborative efforts of this Centre and WHO. This manual is a result of this. The primary objective is to briefly present the existing knowledge about the disease and its pathogen and draw guidelines for procedures for laboratory diagnosis and characterization of leptospires. I hope this document jointly published by the Regional Medical Research Centre (ICMR), Port Blair and WHO will address some of the current issues in leptospirosis diagnosis, surveillance and control.

P. Vijayachari
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<tr>
<td>5-FU</td>
<td>5-Fluoro Uracil</td>
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<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>APPCR</td>
<td>Arbitrarily Primed PCR</td>
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<tr>
<td>CAAT</td>
<td>Cross Agglutination Absorption Test</td>
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<td>DGM</td>
<td>Dark Ground Microscopy</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>EIA</td>
<td>Enzyme Immuno Assay</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immuno Sorbent Assay</td>
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<tr>
<td>EMJH</td>
<td>Ellinghausen McCullough Johnson Harris Medium</td>
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<td>FAFLP</td>
<td>Fluorescent Amplified Fragment Length Polymorphism</td>
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<tr>
<td>IFA</td>
<td>Immuno Fluorescent Antibody Test</td>
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<tr>
<td>IHA</td>
<td>Indirect Haemagglutination Test</td>
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<td>ILS</td>
<td>International Leptospirosis Society</td>
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<td>Lepto-LAT</td>
<td>Latex Agglutination Test</td>
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<td>mAbs</td>
<td>Monoclonal Antibodies</td>
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<td>MAT</td>
<td>Microscopic Agglutination Test</td>
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<tr>
<td>MCAT</td>
<td>Microcapsule Agglutination Test</td>
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<tr>
<td>MSAT</td>
<td>Macroscopic Slide Agglutination Test</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PFGE</td>
<td>Pulsed Field Gel Electrophoresis</td>
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<tr>
<td>RAPD</td>
<td>Random Amplified Polymorphic DNA fingerprinting</td>
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<tr>
<td>REA</td>
<td>Restriction Enzyme nuclease Analysis</td>
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<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
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leptospirosis is an acute bacterial infection caused by spirochetes belonging to the genus *Leptospira* that can lead to multiple organ involvement and fatal complications. It has a wide geographical distribution and occurs in tropical, subtropical and temperate climatic zones. In the developed world most cases that occur are associated with recreational exposure to contaminated water. The incidence seems to be increasing in developing countries. Some countries, where leptospirosis is under surveillance, have recorded this increase in incidence. Most countries in the South East Asia region are endemic to leptospirosis. The International Leptospirosis Society (ILS) made an attempt to compile data on occurrence of leptospirosis in various countries and the data showed that tens of thousands of severe cases occur annually worldwide. This could only be an under-estimate as only a small number of countries participated in the survey and even in those countries, leptospirosis surveillance is far from complete.

A number of leptospirosis outbreaks have occurred during the past few years in various countries particularly in South America and India. Some of these were as a result of natural calamities such as cyclone and flood.

Leptospirosis is considered as the most widespread zoonosis in the world. Leptospirosis affects human beings and many other species of vertebrates. It can present in a wide spectrum of clinical manifestations in human beings. The syndrome of icteric leptospirosis with renal involvement is referred to as Weil’s disease. Another recognized clinical form is that presenting with severe pulmonary haemorrhage. Other complications include acute respiratory failure, myocarditis, meningitis and renal failure. Uveitis has recently been recognized as a late complication of leptospirosis.

Pulmonary haemorrhage is perhaps the most fatal complication in leptospirosis. In Andamans, a significantly higher case fatality ratio has been observed amongst patients who develop pulmonary haemorrhage as compared to patients with other clinical presentations. Serovar Lai belonging to serogroup Icterohaemorrhagiae had been incriminated as a cause of leptospirosis with haemoptysis as the predominant symptom in China and Korea. But other serovars such as Australis have also been found to be associated with similar clinical presentation. Serovars Canicola and Pomona were involved in the 1995 outbreak in Nicaragua and Serogroup Canicola was responsible for an outbreak of leptospirosis with pulmonary haemorrhage in Orissa, India after the super-cyclone in 1999. In Andaman Islands, a few serovars belonging to the serogroup Grippotyphosa including Valbuzzi have been isolated from cases of leptospirosis with pulmonary haemorrhage.

The other most common fatal complication is renal failure. It was observed that a significant proportion of cases with renal failure attending nephrology dept. in Chennai, India had leptospiral etiology. However, in most cases, renal failure is reversed with conservative measures such as maintaining fluid and electrolyte balance and symptomatic therapy. Other complications such as meningitis rarely
Leptospirosis may some times cause intractable hypotension and cardiac arrhythmias and might become fatal.

Leptospirosis, being a zoonotic disease with a large variety of animal species acting as carriers, is difficult to eliminate and perhaps even control in tropical developing countries. The bacteria is 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.
References


In 1886, Adolf Weil described a clinical syndrome characterized by splenomegaly, jaundice, haemorrhages and nephritis. This syndrome is usually referred to as Weil’s disease and this has become synonymous with leptospirosis. 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. In Japan, diseases with traditional names such as nanukayami (seven day fever), akiyami (autumn fever) or hasamiyami (autumn fever in Hasami district) were later proved to be due to leptospirosis. In Europe and Australia diseases with various local names such as cane-cutter’s disease, swine-herd’s disease and Schlammfieber (mud fever) were also later recognized as due to leptospiral infection. Thus, leptospirosis had various names in different parts of the world that denoted seasonal association, symptoms, duration or occupations that were thought to be associated with the disease.

Leptospires were first identified as the cause of Weil’s disease in Japan, where it was common among coal miners. In 1915, Inada and Ido successfully transmitted the infection to guinea pigs and from the blood of the infected animals they grew the responsible organism. Unaware of this development, Huebener and Reiter reported the successful transmission of Weil’s disease to guinea pigs in October 1915. They demonstrated flagella like bodies in Giemsa stained blood smears. Ten days later Uhlenhuth and Fromme also reported similar findings. They also recorded anicteric leptospirosis caused by the same spirochaete for the first time. Several years before this, Stimson had reported the presence of spiral organisms in kidney specimens stained with Levedeti technique (used to demonstrate spirochaets) from a patient. A World Health Organization Scientific Group on Research in Leptospirosis (1962-65) recognized Stimson’s description as the first demonstration of leptospira. Naguchi, who himself was not a physician, grew a spirochaete from the liver specimen of a patient, who, he was told, had died of yellow fever. He named this organism as Spirochaeta icteroids. It closely resembled the causative organism of Weil’s disease and it was not possible to distinguish the two organisms in cross-protection tests in guinea pigs. By 1930, after the discovery of yellow fever virus, it became clear that the two organisms were essentially the same and the patient might have had leptospirosis rather than yellow fever.

The Japanese workers who discovered the organism responsible for Weil’s disease named it Spirochaeta icterohaemorrhagiae. However, there were considerable differences in the appearance and movement of this organism from other spirochaetes. When Stimson observed the organism in the kidney specimens, he had doubts about it being a spirochaete and designated it provisionally as Spirochaeta interrogans on account of its hooked extremities. The Germans Huebener and Reiter called the organism Spirochaeta icterogenes and Uhlenhuth and Fromme as Spirochaeta nodosa. The French, however, adhered to the original nomenclature.
Rodents are the first recognized carriers of leptospires. A large number of studies on seroprevalence and leptospirosis in animals was recognized as an important veterinary problem as well as a source of infection to man. During the period from 1950s to 1970s much data on the ecology of leptospires in tropical countries were generated because of military operations in South East Asia.

Spirochaeta icterohaemorrhagiae of the Japanese authors on the ground that the Japanese workers, being the discoverers of the organism, were alone entitled to name the organism. Naguchi introduced the genus Leptospira in 1917 on account of the difference in morphology and movement. He described the characteristic feature of this organism as ‘long, slender, cylindrical, highly flexible filament with tightly set, regular, shallow spirals’. The family Leptospiraceae among the Order of Spirocheatales was proposed by Pillot and Ryter in 1965.

Soon after the discovery that Weil’s disease was caused by leptospires, several other disease entities were recognized to have a leptospiral etiology. These include ‘nanukayami’ or the Japanese seven-day fever and ‘akiyami’ the harvest fever. The same Japanese group that identified leprospires described the role of rats as their carriers. During the early days, it was considered that there were three types of leptospires i.e. Leptospira icterohaemorrhagiae, L. icteroids and L. hebdomadis, which differed serologically from one another though morphologically they were similar. Leptospires were thought to be responsible for yellow fever, seven-day fever and Weil’s disease and probably for dengue and sand fly fever. Much of the basic current knowledge about leptospires and leptospirosis was understood within a decade of the discovery of leptospires. Several types of leptospires such as L. icterohaemorrhagiae, L. canicola, L. grippotyphosa, L. andamana, L. australis, L. bataviae, L. tarassovi and L. pomona were recognized during this period. By 1940s leptospirosis in animals was recognized as an important veterinary problem as well as a source of infection to man. During the period from 1950s to 1970s much data on the ecology of leptospires in tropical countries were generated because of military operations in South East Asia.

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Initial studies on pathogenesis showed that leptospires are widely distributed in the organs. The studies also showed that leptospires adhere to platelets and this adhesion was thought to be the cause of thrombocytopenia and haemorrhages seen in Weil’s disease. Information about immunity was generated soon after the discovery of the organism and it was understood that immunity is mostly humoral in nature. In the initial publications, an immune agglutination with convalescent sera was described. Soon several serological tests including latex, haemagglutination and other macroscopic agglutination tests were developed. Microscopic Agglutination Test (MAT) developed some 70 years ago still remains as the standard. An ELISA test for the diagnosis of leptospirosis was developed in 1980s. Before the discovery of antibiotics, specific therapy was limited to immunotherapy using rabbit or horse antisera and arsenicals. Since rats were the first recognized animal carrier of leptospires, the initial attempts at control of the disease were centred on rodent control. Once it was
understood that a wide variety of animals can harbour the organism and act as the source of infection to man, the difficulties in control became obvious.

In India, reports of Weil’s disease had started appearing in the literature by the end of 19th century13. Chowdry reported a series of jaundice cases that occurred over a decade starting from 1892 in Andaman, which he believed were cases of malaria, though malaria parasite was not seen in blood smears. Later researchers believed that these were actually cases of Weil’s disease6, 14. Wolley reported a series of 40 cases of severe malaria with jaundice and 17 deaths that occurred among self-supporting convicts of Andamans in 190915.

In this series also, malaria parasite was absent in blood smear. Wolley also reported observing motile rod-like structures attached to RBCs. In 1921 de Castro reported five cases of ‘toxic jaundice or unknown origin’ in Andamans16. He examined blood films for leptospira but failed to demonstrate them. In 1926, Barker reviewed these reports and concluded that these cases were actually cases of Weil’s disease. He also obtained microscopical evidence of presence of leptospires in stained blood films of patients.

In 1931, the first report of bacteriologically confirmed cases of leptospirosis originated from the Andaman islands14. Twenty four isolates of leptospires were obtained within a period of four months from 64 patients with Weil’s disease among the free-living convicts in Port Blair and surrounding villages. Isolated reports of leptospirosis, diagnosed either by demonstration or isolation of the organisms or by serological evidence, appeared regularly from several places including Calcutta, Assam and Bombay17, 18, 19. But after the 40s, reports of leptospirosis were comparatively few till 1980 and most the reports, which appeared during this period, were from the four metropolises.

Earlier, the disease was confined to a few places like the Andamans, Bombay, Calcutta and Madras. During the 80s reports of human leptospirosis started appearing in increasing frequency and in many places, where it had never occurred earlier. Outbreaks have occurred in various places and often with high mortality rates. Though there has not been any systematic compilation of data on incidence of the disease, judged by the number of reports appearing in the literature, the disease is becoming more and more common. Now it has been recognized that the disease occurs in all the Southern states, in West Bengal and Assam in the Eastern Region, in Bihar, Uttar Pradesh and Delhi in the North, in Maharashtra and Gujarat in the West and in the Andaman islands. In many places like the Andamans, Tamil Nadu and Kerala, leptospirosis has been recognized as an important public health problem. Leptospirosis accounts for considerable proportion cases with clinical complications like renal failure and myocarditis20, 21, 22. Considering the epidemic potential of the disease and the nature of the environment and lifestyle of the people, leptospirosis is a constant threat to the health of our people23.
References


Morphology

Leptospires are flexible helical rods that are actively motile. The motility of leptospires is characterized by rotation about its longitudinal axis and flexion and extension. The rotatory movement occurs in both directions alternately. Usually one or both ends are bent or hooked. They are too thin to be seen under light microscope and are best visualized under dark ground microscope. They cannot be stained readily with aniline dyes and can be stained only faintly by Giemsa stain. Leptospires are best stained by silver impregnation techniques\textsuperscript{1-3}.

Microscopic appearance under dark ground illumination

The bacterial cells are thin, finely coiled and actively motile. The size of the cell ranges from 6-20 µm in length and about 0.1 µm in thickness. Under low power magnification (200X) bacterial cell looks like a small piece of thread and hooked ends are visualized as dense dot like structures. The fine coils are seen only under high power magnification (1000X).

Fig. 1. Leptospira under dark ground (Microscope (200X))

Electron microscopic features

The cell of leptospira has several coils. The coils have amplitude of 0.1 µm to 0.15 µm and a wavelength of approximately 0.5 µm. Freshly isolated pathogenic leptospires tend to be shorter and tightly coiled than laboratory strains that have undergone repeated subcultures.

*Leptonema illini* is wider with an amplitude of approximately 0.13 µm and wavelength of 0.6 µm whereas *T. parva* is shorter and tightly coiled (amplitude 0.13 µm - 0.14 µm and wavelength 0.3 µm -0.36 µm). The direction of the coils is right handed (clock wise coiling).

Cultural characteristics

Leptospires are aerobes and utilize long chain fatty acids as carbon and energy sources. In addition to long chain fatty acids, Vitamin B1, Vitamin B12 and ammonium salts are also required for their growth. Leptospires utilize purine bases but not pyrimidine bases and hence they are resistant to the antibacterial activity of the pyrimidine analogue 5- fluro uracil. This compound is used in selective media for the isolation of leptospires from contaminated sources.

Fig. 2. Electronmicrograph of leptospires (45,0000X)
Because of the inherent toxicity of free fatty acids, these must be supplied either bound to albumin or in non-toxic esterified form. Pyruvate enhances the initiation of growth of the parasitic leptospires. Leptospires are grown at 28°C to 30°C with pH in the range of 7-8. At 30°C the generation time varies between 7-12 h and yields of 1-2 X 10^8 cells per ml can be obtained in 7-10 days. The pathogenic leptospires can survive for many days or even months in wet soil and fresh water with neutral or slightly alkaline pH. In salt water the survival time is only few hours.

**Culture media**
A wide variety of culture media can be used for the cultivation of leptospires. The routinely used culture media are described briefly here:

i) **Media that contains rabbit serum:** These include Korthof’s medium, Fletcher’s medium and Stuart’s medium. Rabbit serum contains nutrients including high concentrations of bound vitamin B12 which helps in the growth of leptospires. All these media can be used for the isolation of leptospires from the clinical specimens and for the maintenance of leptospires but not for the preparation of antigens for MAT.

ii) **Fatty acid albumin medium:** In this medium long chain fatty acid is used as a nutritional source and serum albumin as detoxicant. This medium is popularly known as Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and widely used for isolation, maintenance and preparation of antigens for MAT and for growing leptospires in bulk.

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**Fig. 3. Phenotypic classification of leptospires**

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*Leptospires*

**L. interrogans**

Serovars (No. = 250)
E.g. *L. interrogans* serovar *Icterohaemorrhagiae* strain RGA

Serogroups (No. = 25)
E.g. *L. interrogans* serovar *Icterohaemorrhagiae* strain RGA in the *Icterohaemorrhagiae* serogroup

**L. biflexa**

Serovars (No. = 45)
E.g. *L. biflexa* serovar *patoc* Strain Patoc 1

Serogroups (No. = 38)
E.g. *L. biflexa* serovar *patoc* Strain Patoc 1 in the *semaranga* serogroup
iii) **Protein-free medium:** In this medium long chain fatty acids are treated with charcoal to detoxify the free fatty acids which are highly toxic to leptospires. The antigenicity and other characters of the cells grown in this medium are similar to those media described above.

Culture media can be enriched by addition of 1% fetal calf serum or rabbit serum to cultivate the festidious leptospiral serovars. Selective culture media, containing 5-FU 50-1000 µg/ml or a combination of nalidixic acid 50 µg/ml, vancomycin 10 µg/ml and polymixin B sulphate 5 units/ml or a combination of actidione 100 µg/ml, bacitracin 40 µg/ml, 5 FU 250 µg/ml, neomycin sulphate 2 µg/ml, polymixin B sulphate 0.2 µg/ml and rifampicin 10 µg/ml can be used to avoid the contamination.

Liquid medium can be converted into semisolid and solid by the addition of agar or agarose. Semisolid media contain 0.1-0.2% agar whereas solid medium contain 0.8-1% agar. Liquid medium is used for the cultivation of leptospires to be used in various tests and other purposes. Semisolid medium is commonly used for the isolation of leptospires and for maintaining the cultures. Colonies are sub surface and visible at 7-21 days of incubation. Solid medium is not ideal for isolation or maintenance of leptospires and mainly used for the research purpose to clone the leptospires from mixed letospira cultures.

**Classification of Leptospira**

The genera *Leptospira*, *Leptonema* and *Turneria* belong to the family of *Leptospiraceae*. The families *Leptospiraceae* and *Spirochaetaceae* (genera *Spirochaeta*, *Cristispira*, *Borrelia* and *Treponema*) make up the order *Spirochaetales*. The classification and nomenclature of Leptospira is complex. Presently two different classification systems - one based on phenotypic characters and other on the genetic homology are being used.

**Phenotypic classification:** The genus leptospira is subdivided into two species (fig. 3), namely, the *L. interrogans* (pathogenic) and the *L. biflexa* (non-pathogenic). Growth at 13°C and in the presence of 8-azaguanine is being used routinely for speciation. The growth of pathogenic leptospires is inhibited both at 13°C and in the presence of 8-azaguanine whereas non-pathogenic ones grow at 13°C and are resistant to 8-azaguanine. Both the species have several serovars and serovar is the basic taxon, which is defined on the basis of surface antigenic makeup.

"Two strains are said to belong to different serovars if after cross absorption with adequate amount of heterologous antigen more than 10% of the homologous titre regularly remains in at least one of the two antisera in repeated tests". Closely related serovars are arranged into serogroups. However, serogroup designation has no official taxonomic status and is intended for laboratory use.

More than 250 serovars arranged into 25 serogroups have been described under the species *L.interrogans*. The species *L.biflexa* has 65 serovars arranged in 38 serogroups. The binominal classification system is strictly followed. However, serovar and serogroup name may be added e.g. *Leptospira interrogans* serovar icterohaemorrhagiae in serogroup icterohaemorrhagiae (*L.interrogans*, serovar Icterohaemorrhagiae in serogroup Icterohaemorrhagiae) or *Leptospira biflexa* serovar Patoc in serogroup Semaranga (*L. biflexa* serovar Patoc in serogroup Semaranga).

A panel of rabbit antisera (group sera) is used for serogroup determination. Cross Agglutination Absorption Test (CAAT) is the test of choice for serovar determination. A panel of monoclonal antibodies (mAbs) are helpful for comparing antigenic pattern between reference strains and isolates.

**Genetic Classification:** Based on genetic homology in DNA hybridization experiments, 15 genomic species (*L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. inadai*, *L. biflexa*, *L. meyeri*, *L. wolbachii*, *Genomo species 1*, *Genomo species 3*, *Genomo species 4* and *Genomo species 5*) have been described in the genus Leptospira (fig. 4) whereas *Leptonema* and *Turneria*
has one species each (L. illini and T. parva respectively).

Genomic species is a group of Leptospiraceae serovars whose DNAs show 70% or more homology at the optimal reassociation temperature of 55ºC or 60% or more homology at a stringent reassociation temperature of 70ºC and in which the related DNAs contain 5% or less unpaired bases.

The genus Leptopsira is characterized by G+C content of 34.4 mol%. The genus Leptonema has G+C content of 51-53 mol% whereas the G+C content of the genus Turneria is 47-48 mol%. A complete sequencing data is available on two leptospires (serovar Lai and serovar Copenhegeni). Leptospiral genome has two chromosomes - large chromosome (CI) and small chromosome (CII). The size of the large chromosome ranges from 4,332,241 bp to 4,277,185 bp whereas the size of the small chromosome is in the range of 358,943 bp to 350,181 bp.

Though the DNA-DNA hybridization is considered to be the gold standard technique for species level identification of leptospires, it is seldom used because of its complexity. Several PCR based DNA fingerprinting methods have become popular and are being used routinely for characterization of leptospires in recent years. Random Amplified Polymorphic DNA (RAPD) fingerprinting, Arbitrarily Primed PCR (APPCR) and Fluorescent Amplified Fragment Length Polymorphism (FAFLP) are some of the examples. The technical details of the various techniques used for the characterization of leptospires are described in chapter 8.
References


Geographic distribution and magnitude of the problem

Leptospirosis has a wide geographical distribution and occurs in tropical, subtropical and temperate climatic zones. In the developed world, the incidence has come down substantially and most cases that occur now are associated with recreational exposure to contaminated water. In contrast, the incidence seems to be increasing in developing countries. Some countries such as Thailand, where leptospirosis is under surveillance, have recorded an increase in the incidence of leptospirosis. Most countries in the South East Asia region are endemic to leptospirosis. The International Leptospirosis Society (ILS) made an attempt to compile data on occurrence of leptospirosis in various countries. The data shows that on an average 10,000 severe cases requiring hospitalization occur annually all over the world.

Vast majority of leptospiral infections are either subclinical or result in very mild illness and the patients may not seek medical assistance. In a small proportion of patients, severe complications may set in. In such cases, there would be clinical manifestations of multiple organ involvement and the case fatality ratio could be more than 40%. Because of the protean manifestations of leptospirosis it is often misdiagnosed and under-reported.

A number of leptospirosis outbreaks have occurred during the past few years in various places such as Nicaragua, Salvador and Rio de Janeiro in Brazil and Orissa, Mumbai and Andaman Islands in India. Outbreaks usually occur on the aftermath of natural calamities such as cyclones, floods and flash-floods.

Leptospirosis is currently identified as a worldwide public health problem. In endemic areas, leptospirosis is a major cause of various clinical syndromes such as jaundice, renal failure, myocarditis and atypical pneumonia. The annual incidence of leptospirosis increased from 0.3/100,000 persons during the period 1982 - 1995 to 3.3/100,000 persons in 1997 - 98 in Thailand. A multi-centric study in India showed that leptospirosis accounts for about 12.7% of cases of acute febrile illness attending hospitals. Leptospirosis is the cause of a significant proportion of cases of non-hepatitis A - E jaundice, non-malarial febrile illnesses and non-dengue haemorrhagic fever in South East Asian countries.

In many tropical endemic areas, a significant proportion of the population is exposed to leptospires. High seroprevalence rate is found among healthy population of many endemic areas such as Andaman Islands, Seychelles Island, rural areas around Chennai etc. High seroprevalence has also been observed among some of the primitive tribes living in wild.

Leptospirosis is currently identified as a worldwide public health problem. In endemic areas, leptospirosis is a major cause of various clinical syndromes such as jaundice, renal failure, myocarditis and atypical pneumonia.
the jungles\textsuperscript{19}. Even in subtropical and intertropical areas, seroprevalence could be high among certain occupational groups such as farmers\textsuperscript{20,21}. In endemic areas, the incidence of asymptomatic infection could also be very high. A survey conducted in Seychelles\textsuperscript{17} showed 9\% point prevalence of asymptomatic leptospiral infection as proved by positive Polymerase Chain Reaction Test (PCR). In a study conducted in the North Andaman, 27\% of the 396 persons followed up serologically had evidence of leptospiral infection during the follow up period of 12 weeks in the post-monsoon season\textsuperscript{22}.

**Transmission cycle**  
Leptospirosis is primarily an infection of animals, humans being accidental victims. The natural habitat of leptospires is the renal tubules of carrier animals. The animal hosts of leptospires are broadly categorized as carrier hosts, in whom the carrier state is temporary ranging from a few months to years, and reservoir hosts in whom the carrier state is life long. The reservoir hosts are the primary source of leptospires. They infect both animals and human beings. The carrier hosts who get infection either from reservoir hosts or from other carrier hosts can also act as the source of infection to human beings. Man to man transmission is very rare and leptospiral infection in human beings is a dead end of transmission chain. Therefore, the source of infection both in animals and humans is the carrier animal, though infection may actually occur through various environmental vehicles.

Although leptospires are susceptible to various environmental factors, particularly drying, acidic pH, salinity and presence of detergents and other bactericidal agents, they can survive for long periods in water and wet soil.

![Fig. 1. Transmission cycle of leptospirosis](image-url)
under favourable conditions. Some serovars of leptospires have been found to survive retaining their infective potential in soil for up to 74 days. There is also indirect evidence that leptospires can grow and multiply in environment under favourable conditions. Thus environment plays an important role in the epidemiology of leptospirosis.

Fig. 1 is a schematic diagram of the transmission cycle of leptospirosis involving reservoir hosts, carrier hosts, environment and human beings.

Three basic factors i.e. the animal carriers, environment suitable for the survival of leptospires and exposure of people to contaminated environment, carrier animals, their urine, body fluids or tissue, are always involved in the transmission of leptospiral infection. However, the exact nature of these factors varies from one ecological setting to the other, thus making the transmission cycle complex and dynamic. Successful transmission depends on the presence of all these three factors. Fig. 2 shows the transmission triad of leptospiral infection. Successful transmission occurs in the area where all the three factors coexist (shown in blue in the diagram).

Seasonal trends
Occurrence of leptospirosis shows strong seasonal fluctuations in most epidemiological settings. This fluctuation most often reflects seasonal changes in the environment, with peak occurrence corresponding to the season when the environment is most suited for the survival of leptospires. In South East Asian countries, where monsoon is active, the peak occurrence often follows the monsoon, when the land becomes wet and water-logged thus facilitating the survival of leptospires. Fig 3. shows the association of occurrence of leptospirosis with rainfall in Andaman Islands. The peak occurrence always follows the peak rainfall. In contrast, in subtropical and temperate countries, the peak occurrence is often observed during the summer months, when the environment becomes warm and suitable for the survival of the bacteria. Seasonal trends are also affected by occupational or social activities such as harvesting or outdoor activities as part of fairs and festivals.

Carrier animals
Rats are the first recognized carriers of leptospires. They are often incriminated as the source of infection to human beings. A large number of studies on seroprevalence and leptospiral carrier state in rodents have been conducted in various countries. Although serovar Icterohaemorrhagiae has been often associated with rodents, other serovars have also been isolated. Other rodents such as bandicoots, mouse, mongoose and the aquatic rodent coypu of France etc. have also been implicated as the source of infection in different ecological niches.

Several domestic animal species can harbour leptospires and act as source of infection to human beings. Serovars Hardjobovis, Pomona, Bratislava and Grippotyphosa are commonly associated with cattle. Leptospirosis in cattle could be totally unapparent or may result in acute febrile illness, diarrhoea or mastitis. Studies have shown that trans-placental transmission of infection can occur in cattle. Sometimes carrier cattle could be seronegative even to the infecting strain and therefore seropositivity would not be a reliable indicator of carrier state. In endemic areas, a significant proportion of the cattle could be seropositive, which indicates that the exposure level in cattle could be very high and they may play an important role in the transmission of infection.
Pigs are commonly infected with serovars Pomona, Tarassovi, Grippotyphosa, Bratislava, Sejroe and Icterohaemorrhagiae. Adult non-pregnant infected pigs are usually symptom free. In communities where pigs are reared close to human habitation, they can be the source of infection to human beings. Sheep has also been shown to harbour leptospires for prolonged period of time. Serovars Canicola and Icterohaemorrhagiae are commonly isolated from dogs. Infected dogs may suffer illness usually referred as Stuttgart Disease, which is characterized by vomiting, dehydration, bloodstained faeces, mucosal sloughing and death. Surviving dogs may have chronic nephritis and may shed leptospires in their urine for prolonged period of time. Leptospiral seropositivity has been observed in many wild animal species including opossums and sea lions. However, their role in the epidemiology of human leptospirosis is not clear.

**Mode of transmission and risk groups**

Leptospires are ubiquitous. They are found wherever experts in leptospirosis, medical and veterinary practitioners and epidemiologists, who are aware of the epidemiology of the disease and adequate specialist laboratory facilities exist. The primary source of leptospires is the excretor animal, from whose renal tubules leptospires are excreted into the environment with the animal urine. Transmission can be direct or indirect. Direct transmission occurs when leptospires from tissues, body fluids or urine of acutely infected or asymptomatic carrier animals enter the body of the new host and initiate infection. Direct transmission among animals can be transplacental, by sexual contact or by suckling milk from infected mother. Presence of leptospires in genital tracts as well as transplacental transmission has been demonstrated in animals. Direct transmission from animals to human beings is common in occupations that involve handling of animals or animal tissue such as butchers, veterinarians, cattle and pig farmers, rodent control workers etc. Accidental infection to veterinarians has also been recorded.

Indirect transmission occurs when an animal or human being acquires leptospirosis from environmental leptospires, originating in the urine of excretor animals. It is considered that the most common portal of entry of leptospires into the host body is through intact skin. Leptospiral seropositivity has been observed in many wild animal species including opossums and sea lions. However, their role in the epidemiology of human leptospirosis is not clear.
are exposed to wet environments because of occupational or other activities. Leptospirosis is a known health hazard of rice farmers in countries such as Indonesia and Thailand. High incidence of leptospirosis has been recorded in provinces with large populations of farmers. Outbreaks have occurred in Korea on several occasions when the fields were flooded before harvest. Outbreaks have occurred among general population when people are exposed to floodwaters that have high chance of leptospiral contamination.

Leptospirosis has been recognized as a potential hazard of water sports and other recreational activities that expose people to possibly contaminated waters. Outbreaks associated with recreational exposure to water have been reported from several countries.

Ingestion of contaminated water has occasionally been reported to cause leptospirosis by some investigators. Cacciapouti B et al investigated an outbreak of leptospirosis that occurred in a small town in Central Italy. In a case-control analysis, drinking water from a fountain was found to be significantly associated with leptospirosis. Corwin A et al (1990), who investigated an outbreak of leptospirosis at Okinawa, Japan, reported that swallowing water while swimming was significantly associated with leptospirosis. Human-to-human transmission through breast-feeding has also been recorded. However this mode of transmission is not of much epidemiological importance as such instances are rare.

**Risk factors**

Occupational, behavioural or environmental factors that expose people to wet environment that is likely to be contaminated with leptospires as well as factors that expose people to the excretions or tissues of carrier animals pose risk of leptospiral infection. Depending on the environment and the social and occupational behaviour, the factors that pose risk of leptospiral infection may vary from community to community. In a study conducted in North Andaman, various outdoor activities including agricultural work, fishing in fresh water, crossing water bodies on the way and use of stream water for bathing were found to be associated with leptosomal seropositivity. In Hawaii, use of water catchment systems, drinking surface water and presence of skin wounds were found to be the factors associated with leptospiral infection. In another study conducted in Northeastern Thailand, where leptospirosis is a public health problem among the rice farmers, various rice farming activities were found to be independently associated with leptospiral infection. In contrast, the risk factors identified in Seychelles Islands were gardening, staying in corrugated iron house, wet soil around house, refuse not being collected by public service, presence of cats at home, skin wounds and drinking home brews, while indoor occupation was found to be a protective factor. Ashford DA et al studied the risk factors of leptospirosis in El Sauce in Nicaragua and came up with some interesting findings. The only identified risk factors were rural household and gathering wood, while indoor water source was a protective factor. Shelling/husking corn was found to have significant interaction with the type of household, with it being a risk factor in urban households and a protective factor in rural households. In a population based matched case-control study conducted in Salvador, a Northeastern coastal city in Brazil, the identified risk factors were related to exposure to sewage and rats. The variability in the risk factors in different parts stresses the point that the transmission dynamics of leptospirosis varies greatly from one ecological setting to the other.
### Table 1. Risk factors of leptospirosis in different areas

<table>
<thead>
<tr>
<th>PLACE</th>
<th>RISK FACTORS</th>
<th>PROTECTIVE FACTORS</th>
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<tbody>
<tr>
<td>Andaman</td>
<td>Agricultural work, exposure to wet fields, participating in rice harvesting, working in the jungles, other outdoor activity, fishing in fresh water, crossing water bodies on the way, use of stream water for bathing</td>
<td></td>
</tr>
<tr>
<td>Hawaii</td>
<td>Use of water catchment systems, drinking surface water, presence of skin wounds</td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>Walking through water, plucking paddy sprouts for replanting, ploughing, fertilizing in wet field</td>
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<tr>
<td>Seychelles</td>
<td>Gardening, staying in corrugated iron house, wet soil around house, refuse not being collected by public service, presence of cats at home, skin wounds, drinking home brews</td>
<td>Indoor occupation</td>
</tr>
<tr>
<td>Nicaragua</td>
<td>Rural household, gathering wood</td>
<td>Indoor water source</td>
</tr>
<tr>
<td>Brazil</td>
<td>Open sewer in proximity to residence, peridomiciliary sighting of rats, sighting groups of five or more rats, workplace exposure to contaminants</td>
<td></td>
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</table>

**Fig. 4. Targets for control strategies on the transmission cycle of leptospirosis**
In the rural setting it is often the agricultural exposure to wet fields possibly contaminated with the urine of rats or farm animals which is the cause of leptospirosis.

**Epidemiological settings**

The eco-epidemiological settings in which leptospiral transmission occurs can broadly be categorized as urban, rural, recreation associated and disaster sequel. In the urban setting, rats infesting sewage networks, overflowing sewage during rains, flooding of roads and exposure of people to flooded roads create an ideal environment for the transmission of leptospirosis. In the rural setting it is often the agricultural exposure to wet fields possibly contaminated with the urine of rats or farm animals which is the cause of leptospirosis. Leptospiral infection also occurs due to exposure to river or stream water as a result of recreational activities such as canoeing, rafting or swimming. Leptospirosis has now been recognized as a possible sequel of natural disasters such as cyclones and floods as during such times people and animals are exposed to wet environments for a prolonged period of time.

**Prevention and control**

Prevention of leptospirosis essentially is by identifying the source and interrupting the transmission. Intervention strategies can target many points in the transmission cycle of leptospirosis. Fig. 4 summarizes the intervention targets on the transmission cycle and the strategies that could be adopted. In different epidemiological setting, different animal species could be the primary source of infection. Even in the absence of epidemiological evidence of association between contact with an animal species and human leptospirosis, if significant carrier state is detected in animals, it could be taken as a potential source of infection to humans. Several strategies have been described to reduce the load of carrier domestic animals. These include test and slaughter carrier animals, test and treat, vaccination of herds and rodent control measures.

Measures targeting the environment are application of substances to make the environment unsuitable for the survival of leptospires, water-shed management, better irrigation practices etc. Measures targeting carrier animals or environment can only be devised only after clearly understanding the transmission dynamics in a particular community, whereas measures targeting people are independent of the epidemiological variability. Measures targeting human beings include vaccination and chemoprophylaxis. Vaccines have been developed for use on man, however the existence of a large number of serovars of leptospires makes it difficult to develop a universally effective vaccine. Chemoprophylaxis with doxycycline has been tried on soldiers from non-endemic areas visiting endemic areas and was found to be almost 100% effective. However, a study conducted in endemic area during an outbreak showed only 54% protection. Chemoprophylaxis can only be used in outbreak situations or on travellers. Although the basic principles of prevention such as reduction of source, environmental sanitation, more hygienic work-related and personal practices etc. are same everywhere, there is no universal control method applicable to all epidemiological settings. A good understanding of the ecological, epidemiological and cultural characteristics of a community that faces the problem of leptospirosis is the essential prerequisite for evolving an effective and acceptable intervention strategy.
Leptospirosis

References


The clinical manifestations of leptospirosis vary greatly. The incubation period is usually 7-14 days but may range from 2-21 days. Sub clinical and mild infections are quite common. Only a small proportion of cases develop severe leptospirosis. The various clinical manifestations are described in this chapter.

Anicteric febrile illness
The typical leptospirosis is biphasic illness. The first phase which is called as septicemic or leptospiremic phase is characterized by acute systemic infection and by the presence of leptospira in the blood and cerebrospinal fluid. This phase lasts for 4-7 days followed by a 1-3 days of afebrile and asymptomatic period and then the second or immune phase starts with occurrence of fever and presence of leptospira in the urine.

The onset is abrupt with rapidly rising fever, which is associated with chills and rigors, increasing prostration, severe headache and body ache. Temperature ranges from 100-105°F. The headache is severe, persistent, usually frontal, less often retro-orbital but occasionally may be bitemporal or occipital. Body pains are most marked in the lower limbs especially in the calves and thighs. Severe pain in the back, neck, abdomen and upper limbs is frequent. Prostration may be so marked that the patient has to stop work and frequently takes to bed. Anorexia, nausea and vomiting are frequent and may be associated with constipation or diarrhoea. Epistaxis may occur during the early stage. Chest pain, dry cough and haemoptysis may occur. Mental symptoms of restlessness, confusion, delirium and hallucinations and occasionally psychotic behaviour may be prominent features in some patients. Some patients develop acute gastroenteritis with abdominal pain, vomiting and diarrhoea.

The most characteristic findings on examination are conjunctival suffusion and severe myalgia. Conjunctival suffusion occurs in the first three days and lasts from one day to more than a week. It is bilateral, most marked on the palpebral portion and is usually associated with unilateral or bilateral subconjunctival haemorrhage. There is no inflammatory exudate and true conjunctivitis does not occur. Myalgia is severe, even touching the muscle causes intense pain. It is most commonly observed in the lower limbs especially the calves. A transient macular, maculopopular, erythematos, purpuric or urticarial rash may occur, usually on the trunk but it may be localized on the upper limbs or the shins.

The septicemic phase subsides after 4-7 days. The temperature then becomes normal and the patient feels well. The second or immune phase is characterized by severe headache due to meningeal involvement and low grade fever. This lasts for 4-30 days or longer. This biphasic course may not be seen in all patients.

Icteric leptospirosis (Weil’s Disease)
In some patients the septicemic phase instead of subsiding progresses to a severe icteric illness with renal failure. Jaundice is the most
important clinical feature of the severity of illness. Jaundice occurs between the fourth and sixth day but may occur as early as the second day or as late as the ninth day, and deepens rapidly reaching a peak within a week. The liver is often enlarged and tender. Jaundice is due to hepatocellular necrosis, intrahepatic cholestasis and increased bilirubin load from absorption of tissue haemorrhage. Marked elevations of bilirubin with mildly elevated transaminases are characteristics. Death rarely occurs due to hepatic failure. Renal involvement is the most serious complication and is the most common cause of death in icteric leptospirosis. Oliguria occurs in the second week but may occur as early as fourth day of illness. A significant number of patients develop non oliguric renal failure and have a better prognosis than oliguric renal failure. Renal manifestations range form urinary sediment changes (pyuria, albuminuria, hamaturia and granular casts) to severe renal failure. Anorexia and vomiting worsen and hiccups may occur. Meningeal symptoms are frequent but overshadowed by hepatic and renal features. Confusion, restlessness, hallucinations, delusion and convulsions may occur. Severe bleeding, cardiac and pulmonary complications are frequent. Towards the end of the second week the patient is deeply jaundiced, uraemic, and haemorrhagic and become comatose. Death may occur at this stage or early in the third week from renal failure. Occasionally sudden death may occur from arrythmias, cardiac failure or adrenal haemorrhage. Massive bleeding from the alimentary and respiratory tract may also end fatally. Death is rarely due to hepatic failure but is virtually limited to icteric cases and in severe cases the mortality may be as high as 15-40%. In those who are not severely ill, recovery takes place in the second week. Diuresis occurs and the blood urea level falls gradually. Fever subsides and the general conditions improve, however, jaundice takes a longer time to clear.

Haemorrhagic pneumonitis
Pulmonary haemorrhages usually occur in the second week of severe forms of icteric leptospirosis. Occasionally it may occur within 24-48 hours of the onset of illness. The onset is sudden with rapidly raising fever which is associated with headache, generalized body ache, and cough which is dry in the beginning but becomes streaked with blood after two to three days. The patient becomes breathless and toxic. Clinical examination reveals a toxic patient with temperature ranging between 100-105ºF, tachycardia, tachypnoea and hypertension in few. Examination of the respiratory system reveals fine crepitations, which in the initial stage are confined to the bases but soon become extensive involving bilateral lung fields. Massive haemoptysis may cause asphyxiation and death. Mortality in these cases is very high and it may be as high as 50-70% in cases who report late to the hospital. Chest X-ray in these patients shows bilateral alveolar shadowing which appears to be more dense in the mid and lower zones. However the radiological abnormalities may range form a single ill defined opacity, through multiple areas of infiltration to a large area of consolidation. These abnormalities clear up within one week without any residual damage.

Doxycycline has been used as a chemo prophylactic agent for short-term exposure, but it cannot be recommended for routine continuous use or for long-term occupational exposure.
Aseptic meningitis
Leptospiral infection may present as meningitis with fever, headache, photophobia and vomiting and with signs of meningeal irritation (i.e. neck stiffness and Kernig’s and Brudzinski’s signs). The C.S.F. Examination shows a cell count of 10 - 1000 cells/cumm with most of them being lymphocytes and raised protein (10 - 200 mg/dl) with normal sugar. Convulsions, focal neurological signs and encephalitis are rare. Prognosis in the meningitis illness is excellent. Very rarely death may occur form encephalitis. In Leptospiral meningitis, some distinctive features such as myalgia, conjunctival suffusion and evidence of bleeding are frequently present if sought carefully.

Case definition
For the purpose of case detection and surveillance, a working case definition is usually followed. The commonly followed case definition, which is also recommended by the WHO and International Leptospirosis Society prescribes that any person presenting with acute onset of fever, headache and body aches associated with: a) severe muscle tenderness, particularly in calf muscles, b) haemorrhages including sub-conjunctival haemorrhage, c) jaundice, d) cough, breathlessness and haemoptyosis, e) oliguria or f) signs of meningeal irritation should be suspected as a case of leptospirosis and investigated. A suspect, who tests positive in any of the screening test such as dipstick, lateral flow, dri dot or latex agglutination test should be considered as a probable case. Successful isolation of leptospires from clinical specimens, a four-fold or higher rise in titre or seroconversion in paired MAT or a positive PCR is considered as confirmatory evidence of current leptospiral infection.

COMPLICATIONS OF LEPTOSPIROSIS

Myocarditis
Cardiac complications are frequent in leptospirosis. They are usually mild and are observed as electrocardiographic abnormalities ranging from low voltage complexes, non-specific ST and T wave changes, conduction defects and arrhythmias. Atrial fibrillations is the most common arrhythmia. Rare but more severe manifestations are cardiac dilatation, cardiac failure and severe arrythmias from haemorrhagic myocarditis. Sudden death may occur from cardiac failure and arrhythmia. All cardiac abnormalities revert back to normal within 2-3 weeks.

Haemorrhage
Bleeding is a constant feature of leptospirosis and is believed to be due to vascular damage. It is usually mild in anicteric cases but more common and severe in icteric cases. Bleeding may occur from the respiratory alimentary, renal and genital tracts and occasionally into the subarachnoid space and adrenal glands. Death may occur from massive bleeding.

Hypotension
Hypotension is an important complication noted in patient with severe leptospirosis. The causes of hypovolemia are: i) hypovolemia secondary to vomiting, increased insensible water losses and diminished fluid intake, ii) massive haemorrhage most often gastrointestinal, iii) myocardial dysfunction, iv) adrenal haemorrhage, v) widespread vascular injury leading to fluids shifts from intravascular to extravascular fluid spaces, and vi) unidentified vovative endotoxin.

Uveitis
It is a late complication of anterior uveal tract and presets clinically as iritis, iridocyclitis and rarely as chorioretinitis. This may occur in the second week of illness or may be delayed up to one year but is more frequent in the first 6 months. Uveitis may be unilateral or bilateral and the course in variable (i.e acute benign episode, recurrent episodes or a chronic process).
This ultimate prognosis is good but chronic uveitis may cause blindness from cataract formation and hypopion in the anterior chamber.

**Leptospirosis during pregnancy**

The hazards of leptospirosis during pregnancy include intrauterine infection with foetal death and abortion, stillbirth, premature labour and signs of congenital leptospirosis within a week or two of delivery. Leptospores may be secreted in the milk of lactating mothers, who during the septicemic phase should be regarded as potentially infective for breast fed infants.

**DIFFERENTIAL DIAGNOSIS**

Leptospirosis with its varied manifestations may mimic a large number of disease processes.

Anicteric leptospirosis is usually misdiagnosed as P.U.O., viral fever, malaria, enteric fever, influenza or pyelonephritis. The associated features of leptospirosis such as conjunctival suffusion and muscle tenderness if carefully looked for may aid clinical diagnosis. Icteric leptospirosis may be confused with: a) viral hepatitis, b) septicemia with jaundice, and c) malaria. In leptospirosis the onset is abrupt, severe headache, myalgia and conjunctival suffusion are constant features and proteinuria is common, whereas in viral hepatitis onset is gradual, headache and myalgia are mild and proteinuria and conjunctival suffusion are absent.

Haemorrhagic pneumonitis can be confused with bacterial pneumonitis, pulmonary tuberculosis and military tuberculosis. In haemorrhagic pneumonitis the onset is quite sudden with high fever, headache, body ache, conjunctival suffusion and muscle tenderness, patient looks quiet toxic and is breathless. In bacterial pneumonitis the toxicity is not much and conjunctival suffusion and muscle tenderness are absent. Pulmonary tuberculosis and military tuberculosis have an insidious onset and the toxicity, breathlessness, conjunctival suffusion and muscle tenderness are not seen. In leptospirosis urine examination will show proteinuria which will not be found in other conditions mentioned above. The chest X-ray in haemorrhagic pneumonitis due to leptospirosis will show bilateral alveolar shadowing with clear apices which differentiates it from tuberculosis and bronchopneumonia.

Aseptic meningitis has to be differentiated from bacterial and viral meningitis and encephalitis. Bacterial meningitis can be confirmed by CSF examination. Viral meningitis is indistinguishable from Leptospiral meningitis because CSF examination in both will show lymphocytes with raised protein and normal sugar. Conjunctival suffusion, myalgia and evidence of bleeding suggests the diagnosis of leptospirosis and this can be confirmed by serological tests. In children clinical features that are not seen or rare in adults such as hypertension, a calculus cholecystis and pancreatitis can occur.

**TREATMENT**

Antibiotic treatment is effective within 7 to 10 days of infection and it should be given immediately on diagnosis or suspicion. The antibiotic of choice is benzyl penicillin by injection in doses of five million units per day for five days. Patients who are hypersensitive to penicillin may be given erythromycin 250 mg four times daily for five days. Doxycycline 100 mg twice daily for 10 days is also recommended. Tetracyclines are also effective but contraindicated in patients with renal insufficiency, in children and in pregnant women.

Doxycycline has been used as a chemo prophylactic agent for short-term exposure\(^4\), but it cannot be recommended for routine continuous use or for long-term occupational exposure\(^5\).
References


leptospirosis cannot be diagnosed based on clinical grounds alone due to the variability in clinical manifestations, similarity of signs and symptoms with those of other bacterial, viral and parasitic infections and frequent occurrence of the disease in atypical forms. Confirmation of the diagnosis requires laboratory support. However, laboratory diagnosis of leptospirosis is an area not often well understood by many of the workers involved in leptospirosis diagnosis and surveillance. Although an array of tests has been described, availability of laboratory support, in practice, is still a problem. Selection of the right specimens and tests and correct interpretation of test results are important. To understand these points rightly one should have basic knowledge regarding the sequence of events that occur in the host after entry of organism and antibody response against leptospires.

A small number of organisms can cause infection. The incubation period usually ranges from 7-10 days. Leptospirosis may follow a biphasic course. During the first 10 days, there is a phase of leptospiraemia when the leptospires multiply in blood and spread to different organs. The chances of recovery of leptospires from blood or other tissues or body fluids is usually high during this stage. This phase is followed by immune phase or leptospiruria phase when the organisms are excreted in the urine. In this phase, chances of recovery of organisms from the blood is low. The ideal specimen for isolation or demonstration of leptospires during immune phase is urine.

The antibodies usually develop within 2-12 days after the onset of illness. IgM antibody starts appearing early in the course of the disease and reaches detectable levels within one week or as early as on third or fourth day of illness. They reach peak levels during third or fourth week and then decline slowly over months and become undetectable within six months. Rarely IgM may persist at low level for several years.

In a small proportion of patients (about 10%) IgM antibodies may not develop at detectable level and the first appearing antibody may be
IgG. Hence the sera from these patients may give negative results in IgM based immuno assays. IgG antibodies appear later than IgM and reach peak level after few weeks of illness. IgG antibody may persist at low level for years. Microscopic agglutinating antibodies usually appear in detectable level at the end of the first week of illness and reach peak levels during third or fourth week and then decline slowly over months and may become undetectable within years or may persist at low level even for decades.

In about 10% of patients microscopic agglutinating antibodies appear at detectable levels only after about a month of illness. Hence the sera collected from these patients during the first month of illness may give negative result in Microscopic Agglutination Test (MAT). Serovar-specific antibodies are long-lasting than serogroup specific antibodies. In less than 10% of patients the immune response is erratic and antibodies (IgM and microscopic agglutinating) may not appear at detectable level. Hence negative test results observed in immuno assays do not necessarily rule out the disease among a small proportion of patients.

Laboratory diagnosis is broadly divided into two categories- those that give direct evidences and those that give indirect evidences. The direct evidences include either demonstration of leptospires or its DNA and isolation of organism from clinical specimens. Detection of specific
antibodies to leptospires (serological diagnosis) is indirect evidence.

Though several techniques have been described, the most often used procedures and their advantages and drawbacks are mentioned here.

**DARK GROUND MICROSCOPY**

Dark Ground Microscopy (DGM): Demonstration of leptospires by using DGM appears to be a simple and rapid procedure. In old text books it is mentioned that DGM is a useful tool for the diagnosis of leptospirosis but it is not true in practice. Though the organism is present in the blood during acute stage of the disease, the concentration is too low to allow detection by direct microscopy. The leptospiral shedding in urine is intermittent. Moreover serum proteins or cell fragments may mimic leptospires. Even experienced personnel may be confused with these artefacts as in majority of the clinical samples leptospires may not exhibit typical motility due to reactive antibodies or due to mechanical injury during the process of specimen for examination. Critical evaluation of this technique as a diagnostic tool has shown that the test has low sensitivity (40.2%) and specificity (61.5%). The usefulness of differential centrifugation was limited and the motility of the organism further reduced after centrifugation at higher g.

Reading the results is always subjective as in the majority of the samples the number of organism per field ranges from 0-2 and there was always doubt about typical motility. Results obtained in DGM on samples from confirmed patients and controls were identical. Therefore DGM is not recommended as a sole diagnostic tool for the diagnosis of leptospirosis.

**Specimens**

The specimens should be taken aseptically and sent to laboratory without delay, they must not be frozen. Oxalate, citrate or heparin may be used as anticoagulant for blood or pleural fluid.

**Principle**

The principle of dark ground microscopy is that the object is illuminated only by light rays that

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**Fig. 4. Laboratory diagnosis — Direct and indirect evidences**

<table>
<thead>
<tr>
<th>Laboratory diagnosis</th>
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<tbody>
<tr>
<td><strong>Direct evidences</strong></td>
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<tr>
<td>Demonstration of leptospires or its DNA or isolation of organism</td>
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<tr>
<td><strong>Microscopy</strong></td>
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<td>Dark Ground Microscopy</td>
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<td>Phase contrast Microscopy</td>
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<td><strong>Indirect evidences</strong></td>
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<td>Demonstration of antibodies to leptospires</td>
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<td><strong>Serovar specific test</strong></td>
</tr>
<tr>
<td>Microscopic agglutination test</td>
</tr>
</tbody>
</table>
are scattered by the object. The specimen is illuminated only by rays so oblique that unless they are scattered by the object with a refractive index different from that of the suspending medium the rays fail to enter the objective and reach the eyes.

Procedure (Blood)
- Centrifuge 5 ml of blood (treated with an anticoagulant) at 1000 g for 15 min.
- Add approximately 10 µl of plasma on a thin microscopic slide and apply cover slip.
- Examine under dark field microscope with low power and high power (X 200 and X 400).
- If no leptospires are seen, centrifuge the plasma at 3000-4000 g for 20 min.
- Carefully remove the supernatant and examine a drop of sediment microscopically as above.

Procedure (Urine)
- Centrifuge a portion of freshly voided urine at 3000 g for 10 min.
- Examine a drop of deposit by DGM (X 200 and X 400).

Advantages
- Laboratories, where the facilities for the other tests are not available, can undertake this technique. But the results should be confirmed with other standard tests.

Disadvantages
- Low sensitivity and specificity.
- Serum proteins and fibrin strands in blood resembles leptospires.
- The concentration of organism is frequently too low in the specimens.
- Requires technical expertise.
- Equivocal results.

STAINING TECHNIQUES
Various silver impregnation techniques are used for the staining of leptospires in body fluids and tissues. However these techniques have similar limitations as DGM. Therefore these staining procedures are not recommended for direct diagnosis of leptospirosis.

Fontana Staining

Procedure
- Treat the film three times, 30 seconds each time, with fixative.
- Wash off the fixative with absolute alcohol and allow the alcohol to act for 3 min.
- Drain off the excess of alcohol and carefully burn off the remainder until the film is dry.
- Pour on the mordant, heating till steam rises, and allow it to act for 30 sec.
- Wash well in distilled water and again dry the slide.
- Treat with ammoniated silver nitrate, heating till steam rises, for 30 sec, when the film becomes brown in colour.

Fig. 5. Culture stained with Fontana Staining technique
Wash well in distilled water, dry and mount in Canada balsam. 

It is essential that the specimen be mounted in balsam under a cover slip before examination, as some immersion oils cause the film to fade at once. The spirochetes are stained brownish-black on a brownish-yellow background.

**Stock Solutions**

**Silver nitrate solution**
- Silver nitrate 10 gms. in 1000 ml distilled water. Place in an amber coloured bottle with glass stopper and store in refrigerator.

**Alcoholic gum mastic**
- Gum mastic 2.5 gms. in 100 ml absolute ethanol. Dissolve by stirring and leave to stand overnight, filter several times until clear.

**Silver nitrate and sodium potassium tartrate**
- Silver nitrate 2 gms., sodium potassium tartrate 1.65 gms in 1000 ml distilled water. Dissolve the silver nitrate in boiling distilled water. Quickly add the sodium potassium tartrate until the white precipitate changes to gray. Filter while hot into an amber coloured bottle and store in refrigerator.

**Hydroquinone solution**
- Hydroquinone 1 gm in 60 ml double distilled water.

**MODIFIED STEINER AND STEINER TECHNIQUE**

**Procedure**
- Six to seven μm thick sections from formalin fixed tissue blocks embedded in paraffin should be cut and mounted on slides with egg albumin.
- De-paraffinize and hydrate in the usual manner.
- Immerse in a 1.0% aqueous solution of silver nitrate for 2 hours at 56-58°C in an oven.
- Wash thoroughly in distilled water.
- Dehydrate up to absolute alcohol and place the slides in 2.5% of alcoholic gum mastic solution for 5 min.
- Transfer from the gum mastic solution into a reducing solution of hydroquinone (1 gm hydroquinone dissolved in 60 ml distilled water, to which are added 20 ml of a 2.5% alcoholic gum mastic solution and 20 ml of silver nitrate and sodium potassium tartarate solution). Mix the three solutions thoroughly by moving the rack with the slides up and down for a few seconds in the dish. Leave the slides in the reduction mixture for 12-15 min. until the colour of the sections changes to light brown.
- Wash 3 times in distilled water, dehydrate, clear in xylol, and mount in neutral mounting fluid.

The spirochetes appear with a black mirror like contrast distinguishable from the yellow or brown tissue elements.

**ISOLATION OF LEPTOSPIRES**

Isolation of leptospires from clinical specimens is the strongest evidence for confirmatory diagnosis. Isolation and identification is the method of choice to identify circulating serovars in a particular geographical region. In addition locally isolated and identified strains will be more useful to be used as antigens in MAT as local strains were found to be more sensitive and strongly reactive than reference strains. Moreover the local strains will be used for the development of vaccine. However the technique has several drawbacks. Leptospires are slow growing organisms and require several days or weeks to yield cultures and weeks to months for identification. Prior administration of antibiotics greatly reduces the chances of successful isolation.

**Blood Culture**

**Ideal time**: Within 10 days of the onset of symptoms.

**Media**: EMJH and Fletcher’s media.

**Procedure**
- Swab the area with spirit.
- Draw blood using sterile syringe and needle by vein puncture.
- Inoculate two and four drops into two tubes containing 5 ml medium.
- Incubate at 30°C for 4-6 weeks.
Leptospirosis

- Examine the culture using dark field illumination initially on first, third and fifth days followed by 7-10 days interval up to 6 weeks.

Selective culture media containing 50-1000 µg/ml of 5-FU or a combination of nalidixic acid 50 µg/ml, vancomycin 10 µg/ml and polymixin B sulphate 5 units/ml or a combination of actidione 100 µg/ml, bacitracin 40 µg/ml, 5-FU 250 µg/ml, neomycin sulphate 2 µg/ml, polymixin B sulphate 0.2 µg/ml and refampicin 10 µg/ml can be used to avoid the contamination.

Subculture should be made within 48 hours to minimize the inhibitory effect of the selective agents on leptospires.

Growth of the fastidious isolate is encouraged by adding to the medium 0.1% to 0.15% agarose and 0.4% to 1% of rabbit serum or fetal calf serum.

URINE CULTURE

**Time**: 10-30 days after the onset of the disease.

**Media**: Same as above.

**Procedure**
- Collect a sample of midstream urine (freshly voided midstream urine within 2 hours is the ideal specimen).
- Dilute the urine as follows using sterile test tubes and sterile phosphate buffer pH 7.2:
  - Add 0.4 ml of urine to 3.6 ml of PBS pH 7.2 (1 in 10).
  - Add 3 ml of (a) to 3 ml of PBS (1 in 20).
  - Add 2 ml of (b) to 2 ml of PBS (1 in 40).
  - Add 1 ml of (c) to 1 ml of PBS (1 in 80).
- Each resulting 0.5 ml dilution of above is inoculated into 4 separate 5 ml volume of medium.
- Label the tubes mentioning the dilution.
- Incubate at 30°C.
- Examine the culture using dark field illumination at an interval of 7-10 days up to six weeks.

The above procedure should be repeated two or three times with urine samples collected on different days to increase the probability of isolation.

Urine can be filtered (through 0.22 µm filter) and/or inoculated into selective culture media to avoid contamination.

*Urine may have acidic pH in many cases. Therefore urine should be collected in tubes containing equal amount of PBS with pH 7.2.*

CSF CULTURE

**Time**: 5-10 days of the onset of the disease.

**Medium**: As above

**Procedure**
- Inoculate 0.5 ml of CSF into 5 ml of culture media.
- Follow the same procedure as blood culture.

**Advantages of isolating leptospires from clinical specimens**
- Definite proof of infection.
- Circulating serovars can be identified.
- Local isolates can be used as antigens in MAT.
- Local isolates can be used in vaccine development.

**Disadvantages**
- Fastidious organism requires special medium for isolation.
- Leptospires grow slowly. Isolation of leptospires from clinical specimens takes several days to several weeks.
- The technique is laborious, time consuming and is not possible in small laboratories.
- Contamination of culture media by other micro-organisms or by saprophytic leptospires is common in routine practice.
- The successful isolation rate is less due to prior use of antibiotics, imperfectly cleaned glass ware or wrong incubation temperature and pH.
ANIMAL INOCULATION
Clinical materials may be inoculated into laboratory animals. Leptospires in the clinical materials may cause disease in the animals, if they are susceptible. Leptospires can be observed directly in the peritoneal fluid or cultured from the peritoneal fluid, blood or tissues.

Animals used
- Hamsters (4-6 weeks old)
- Guinea Pigs (150-175 gms.)

Procedure
- The inoculation of blood and/or urine is done intra-peritoneally (0.5-1.0 ml)
- From the third day after inoculation, peritoneal fluid is examined under Dark Field Microscope for the presence of leptospires.
- The examination should be done during 3-10 days after inoculation.
- When the inoculated animals die, it is possible to isolate leptospires from liver and kidneys.

Animal inoculation is not recommended due to ethical reasons. Maintenance of animals is very expensive.

MACROSCOPIC SLIDE AGGLUTINATION TEST (MSAT)
The basic principle of the test is similar to other slide agglutination tests used in other infectious diseases such as enteric fever or brucellosis.

There can be different ways to prepare the antigens. One may either use a single serovar or multiple serovars to prepare the antigens. When multiple serovars are used as antigens, antigens can be pooled. Since the technique will require lot of laboratory work, generally a single non-pathogenic serovar patoc (strain Patoc 1) is used.

Preparation of the Antigen
- Leptospires are grown in liquid medium preferably EMJH. Tubes with 3 ml medium are inoculated with the few drops of a 5-7 days old leptospira culture. The inoculated tubes are incubated at 30°C. Cultures are examined by Dark Field Microscope (DFM). If they are satisfactory the cultures are dispensed into an Erlenmeyer flask containing 50 ml of EMJH medium and then incubated for 5-7 days at 30°C in a shaking incubator.
  - Formalin is added to final concentration of 0.5% and the cultures are allowed to stand for at least 30 min.
  - The cultures are centrifuged for 30 min at 10,000 g.
  - The supernatant is discarded and the tubes are allowed to dry in a slanted position for two hours.
  - The sediment is re-suspended in 1.5 to 2 ml of solution containing 0.5% formalin, 12% NaCl and 20% glycerol in distilled water and mixed 15-20 times by sucking up and down with a 2 ml syringe through a 18-20 gauge needle.
  - The antigen is kept at 4°C overnight.
  - Measure the optical density at 520 nanometers in a spectrophotometer. Adjust to a range of 0.550-0.600 by diluting suspension with the NaCl - Formalin-Glycerol solution.
  - Standardize the antigen using control sera.
  - Store at 4°C.

Procedure
- Place 10 µl serum on a glass slide. Add one drop (50 µl) of antigen and mix thoroughly with a applicator stick.
- Place the slide on the mechanical rotator and rotate at 120 rpm for 4 min and read the reaction with an indirect light against a black background.

Recording the results
The reaction is recorded as ++ when the clumps are large and definite, + when the smaller clumps are well defined but the suspension is not clear, +/- when fine clumps are visible but the suspension is not clear and negative when the mixture in the drop is unchanged. Agglutination of + and ++ is considered as positive.
The test is easy to perform and read. The antigen is broadly reactive and stable for six months at 4°C to 8°C. It is more sensitive than Microscopic Agglutination Test (MAT) in the early stage of the disease. However a high percentage of false positive reactions are observed, probably due to lack of standardization and quality control of the antigen preparation. The number of false negative reactions are comparatively low.

**ENZYME IMMUNO ASSAY (EIA)**

EIA is one of the techniques commonly used for the diagnosis. The test can detect specific antibodies earlier than MAT. The advantage of the test is that it can differentiate between recent and past infection by detecting the type of antibodies (IgM or IgG) present in the clinical specimen. In this test, broadly, reactive antigen is used. The antigen antibody reaction is visualised or measured by spectrophotometer/ELISA reader using a conjugate (enzyme conjugated to anti-IgM or IgG) and a colour reagent. The main drawback of the test is it should be standardized locally to find out cut off titre or significant titre for current infection as once a man is infected with leptospires, IgM may persist at low level for several years.

**Preparation of the antigen for the EIA**

- Leptospira interrogans serovar Copenhageni strain Wijnberg or L. biflexa serovar Patoc strain Patoc I is grown in EMJH medium for 10-12 days at 30°C in shaking incubator. Abundant growth is necessary to produce a good antigen.

- If necessary, add 5 ml of sterile diluted Tween 80 (1:10) to 500 ml culture under sterile conditions 4-5 days after inoculation. Incubate for another 5-6 days.

- Before harvesting, check for abundant growth and purity. Also check for the absence of Tween. This can be done by adding formalin to a sample from the culture to get a final concentration of 0.5%, centrifuging at 10,000 g for 30 min and heating the supernatant in a glass tube in a boiling water bath. If the supernatant becomes turbid, not all the Tween has been consumed. The turbidity disappears quickly upon cooling. If there is turbidity, leave the cultures a few days longer in the shaking incubator until all of the Tween has been used up.

- After ensuring the quality of the culture and absence of Tween kill the leptospires with formalin (0.5% final conc.).

- After one hour heat the killed leptospira culture in boiling water for 30 min, shaking every 5 min.

- After cooling to room temperature, centrifuge the culture for 30 min. at 10,000 g. Centrifugation at lesser g is also possible. The supernatant is used as antigen.

- In the liquid form, the antigen is stable for years if stored at 4°C.

**Coating of the ELISA Plates**

- 100 µl of antigen is pipetted into every well of the ELISA plate. A sample of each batch of plates should be checked before being used for routine diagnosis.

- The plates are left at room temperature until complete evaporation of the fluid has taken place (1-3 days, depending on temperature). Avoid exposure to sunlight.

- The coated plates are kept in a closed box, or in sealed plastic bags, at room temperature. The antigen is stable for several years.

**Procedure**

- Rinse the coated plates four times with PBS/Tween.

- During the last two washings leave the fluid in the wells for one minute.

- Dry the plates by tapping them on a cloth or filter paper.

- Make doubling dilutions of the patients’ and controls’ sera in PBS/Tween/BSA. Fill the second column of the wells with 190 µl of dilution fluid, the other wells with 100 µl. Add 10 µl of patients’ and controls’ serum to the second column (dilution is now 1:20). Transfer, after mixing thoroughly, 100 µl to the 3rd well, etc. Discard the final 100 µl. Use the first column as blank.
Cover the plate with another microtitre plate and incubate for one hour at 30°C.

After one hour wash the plate as under 1.

Add to all wells 100 µl conjugate, diluted in PBS/Tween/BSA. For each of the conjugates an optimal dilution should be used (i.e. block titration).

Incubate for one hour at 30ºC as before.

Discard the contents and wash four times as above.

Add to all wells 100 µl substrate.

Leave at RT for two hours.

**Equipment required**
- Micropipettes (Multi & Uni Channel)
- Microtips
- Pipettes

**Comments:** A titre of 1:40 in non-endemic areas and a titre of 1:80 in endemic areas is considered as significant titre for leptospirosis in early stage.

**MICROCAPSULE AGGLUTINATION TEST (MCAT)**

The test is based on the principle of passive agglutination and employs carrier micro-capsule particles on the surface of which ultrasonicated leptospiral antigens are absorbed.

**Kit**
The kit contains two vials of lyophilized reagents viz reagent A and reagent B and one vial of diluents, a test tube rack with mirror for reading the results, test tubes (small) and disposable loop (1 µl). Reagent A incorporates antigens of L. australis, L. autumnalis and L. hebdomadis and reagent B incorporates that of L. canicola, L.icterohaemorrhagiae and L. pyrogenes.

**Procedure**
- Add 2.3 ml of reconstituting solution to the vials of A and B.
- Place 0.3 ml of reagent A into one test tube and 0.3 ml of B reagent into another.
- Using a loop for 1 µl take about 1 µl, of the test serum and place it into the test tube containing reagent A and B separately.
- Shake the test tubes and allow to stand them in the test tube rack with mirror.
- After three hours read the results.

**Advantages**
- Detects IgM antibodies earlier than MAT.
- Single antigenic preparation can be used.
- Heat stable antigens which are stable at room temperature for long periods.
Reading the results
The results are recorded as follows - positive when the agglutination pattern covers the entire bottom of the tube, doubtful when agglutination pattern is slightly larger than the negative control and negative if no agglutination is observed.

Advantages
- Simple to perform and easy to read.
- Does not require any special expertise or equipment.

Disadvantages
- Not a confirmatory test.
- Kits have to be imported.
- Costlier.

Interpretation of the results
a) Positive: Development of clearly visible reddish coloured Ag band (1+, 2+, 3+, 4+).

b) Negative: No development of reddish coloured Ag band.

LEPTO LATERAL FLOW
Lepto lateral flow is based on the binding of specific IgM antibodies to the broadly reactive heat extracted antigen prepared from non-pathogenic Patoc 1 strain. IgM antibodies bound to the broadly reactive antigen are detected with an anti-human IgM gold conjugate contained within the test device.

Kit
- Reconstitution fluid (vial A)
- Lyophilized detection reagent (vial B)
- Dipstick fluid (vial C)
- Dipsticks containing, on a white strip, a leptospira antigen (lower band) and an internal control band (upper band)

Equipment
- Test tubes
- Test tube holder
- Micropipettes (5-250 µl)
- Disposable pipette tips

Fig. 6. Positive and negative results in MCAT
Procedure
- Add five ml of undiluted serum or 10 µl of whole blood added to the sample application.
- Add 130 µl of sample fluid (diluent).
- Wait for up to 15 min.

Reading results
- If only the control band became stained, the test is negative.
- If both test and control bands became stained, the test is considered as positive.

Advantages
- Very quick.
- Both serum as well as blood can be used to perform the test.

Disadvantages
- Expensive.

Fig. 7. Lepto lateral flow, positive and negative test results

LEPTO DRI-DOT

Principle
The Lepto Dri-Dot consists of coloured latex particles activated with a broadly reactive leptospira antigen that is dried onto an agglutination card. The assay is based on the binding of leptospira-specific antibodies to the leptospira antigen. The broadly reactive antigen ensures the efficient detection of a wide spectrum of leptospira infections. The assay is performed by the addition of 10 µl of a freshly prepared serum sample to the dried latex particles. The latex particles are then suspended by mixing with the serum sample using a special spatula followed by swirling of the suspension by hand rotating of the agglutination card. Serum, which has been stored frozen, can be used as well. Agglutination occurs within 30 to 60 seconds and is clearly visible by the formation of fine aggregates that tend to settle at the edge of the droplet.

Kit
- Lepto dri dot cards
- Disposable plastic spatulas

Equipment
- Micropipettes (10 µl)
- Disposable pipette tips

Procedure
- Remove a Dri-Dot card from the packaging and place the card on a bench top with the blue dot facing upwards.
- Spot 10 µl serum next to, but not onto, the blue dot and within the area marked by the black circle.
- Take hold of the plastic spatula with the flat site of the tip facing downwards. Hold the spatula with the thumb and forefinger close to the flat end of the spatula. Suspend serum and blue dot with a quick circular motion while pressing the flat end of the spatula firmly on to the dot. Don’t spread the suspension outside the area marked by the black circle. Proceed with the next step when the blue dot is fully suspended and a homogenous suspension is obtained.
- Keeping the card near horizontally, slowly rotate the card swirling the liquid in circular motion within the limits of the marked areas in order to mix latex and serum sample further and to induce agglutination.
- Read results within 60 seconds.

Advantages
- Simple to perform and easy to read.
- Doesn’t require any special expertise or equipment.
- The Dri-Dot have long shelf lives even at room temperature.
Disadvantages
- Although the results of the Lepto Dri-Dot are found to be in agreement with those of IgM ELISA, neither 100% sensitivity or 100% specificity is claimed.
- It is advised that the Microscopic Agglutination Test to be used as a confirmatory test.
- Kits have to be imported.
- Costlier.

LEPTO-LAT
Lepto-LAT is a latex agglutination test for detection of antibodies to leptospires in body fluids.

<table>
<thead>
<tr>
<th>Materials provided</th>
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<tbody>
<tr>
<td>1. Sensitized latex beads in suspension</td>
</tr>
<tr>
<td>2. A positive control</td>
</tr>
<tr>
<td>3. A negative control</td>
</tr>
<tr>
<td>4. Plastic sticks for mixing</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials required but not provided</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Clean glass slides (Please do not use already used ones)</td>
</tr>
<tr>
<td>2. Micro pipette (5 μl - 10 μl)</td>
</tr>
<tr>
<td>3. Micropipette tips</td>
</tr>
</tbody>
</table>

Principle
Lepto LAT is a agglutination immuno assay for the detection of leptospirosa specific antibodies. The blue coloured latex beads are sensitized with the broadly reactive and specific antigen prepared from pathogenic leptospires and suspended in storage buffer. When the specimen is mixed with suspended latex, antibodies present in the specimen interact with the antigen that is coated on the surface of the latex particles leading to the formation of fine and clearly visible granular agglutination within 60 seconds.

Procedure
- Shake well the vial containing coloured latex beads.
- Transfer 10 ul of latex beads on a slide.
- Add 5 μl of serum near to the latex.
- Mix the serum thoroughly with the latex beads and spread uniformly in circular fashion. (The diameter of the circle should not be more than 1 cm).

• Rotate the slide slowly and gently.
• Record the results within 60 seconds.

Fig. 8. Lepto LAT, positive and negative test results

Interpretation of the test results
Fine clearly visible agglutination occurs within 30-60 seconds. The intensity of the agglutination depends on concentration of the antibodies in a serum sample. Clearly visible granular agglutination is indicates the presence of specific antibodies to leptospires. In stronger reactions fine granular clumps tend to settle at the edge of the circle. Agglutination that occurs beyond 60 seconds may be due to evaporation and should not be considered.

Storage
The coated latex particles are stable up to one year at 4ºC - 8ºC but can be stored for up to six months at room temperature (32-37ºC).

Advantages
- Simple to perform and easy to read and doesn’t require any special equipment or expertise
- The Lepto-LAT have long shelf life even at room temperature
- Cost effective.

Disadvantages
- Results need to be confirmed.

MICROSCOPIC AGGLUTINATION TEST (MAT)
The most commonly used serological technique for confirmation of diagnosis is MAT. The test is highly sensitive when performed on paired sera (acute and convalescent ) and is serovar/serogroup specific. One of the critical issues
of MAT is the cut-off or significant titre for diagnosis, when the test is done only on a single sample. Ideally MAT should be performed on paired sera collected during acute and convalescent stage of the disease to find out sero-conversion or four-fold rise in titre, which is the evidence of current or recent infection. However, collection of convalescent serum sample is difficult in routine practice. It also delays the diagnosis. The alternative is to determine cut-off or significant titre for MAT on a single sample for diagnosis. The cut-off titre for single MAT depends on the baseline titre in the community in a particular geographical region. Different laboratories use different cut-off titres ranging from 1 in 100 to 1 in 400 for diagnosis based on endemicity or baseline titre in the community. Several investigators usually consider a titre of 1 in 100 as a significant titre for diagnosis without considering the endemicity or baseline titres in the community. This may result in over-diagnosis and overestimation of disease burden.

The optimal cut-off titre is assessed by carrying out a baseline study on distribution of titres in the community as well as among confirmed patients. Using this data it is possible to estimate the sensitivities and false positivity rates at different cut-off titres. The titre that gives the lowest number of false results is then chosen as the optimal cut-off titre. This titre can be found out by plotting false positivity rates against sensitivities at different titres. Such a plot is called the Receiver Operating Characteristics (ROC) curve. The titre that lies closest to the left upper corner of the plot will result in least number of false results and hence is considered as the optimal one. Figures 11 and 12 show ROC curves for MAT during first week and second to fourth weeks of illness in endemic and non-endemic areas. In an ROC curve, a straight line drawn from left lower corner to right upper corner is called the no-benefit line, because if a titre that lies below this line is chosen as cut-off the test result doesn’t provide any information useful for diagnosis.

Fig. 9. ROC curve of MAT cut-off titres in endemic area showing that a titre of 1 in 200 is the optimal during 2-4 weeks and no titre is optimal during first week
Although MAT has several drawbacks, it is an indispensable technique in diagnosis and in seroepidemiological studies. The test is more specific and sensitive if performed on paired sera. However, this is always not true and therefore, one should be careful while interpreting MAT titres. Due to cross reactive antigens among serovars belonging to different serogroups or due to the lack of homogeneity within the serovars of certain serogroups, MAT titres may not always provide actual information about infecting/circulating serogroups.

The usual method for carrying out MAT is to mix equal volumes of series of serum dilutions and leptospira culture in the wells of microtitre plates. The serum antigen mixture is allowed to react for a certain period at certain temperature. The degree of agglutination and endpoint titre are determined by examining a drop of the mixture by dark field microscopy. The degree of agglutination as such may be difficult to observe and the degree of agglutination is often assessed by determining the number of free non-agglutinable leptospires. Hence, there is an element of subjectivity in reading the test results.

**Panel of antigens**

A battery of antigens, covering the range of serovars that are expected or likely to be circulating in a particular geographical area, where the patient becomes infected, should be used. Locally isolated strains should be included in the panel, if possible, as they may give more specific and sensitive results than reference strains. At least one strain of saprophytic serovar (Patoc I) should also be included in the panel to act as genus specific antigen to detect infections caused by serovars/strains not yet known to exist in a particular geographical area. The recommended panel of antigens (one strain representing each known serogroup) may be used while testing sera received from a geographical area where the information about circulating serovars is lacking. The recommended panel is given below.

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**Fig. 10. ROC curve of MAT cut-off titres in non-endemic area showing that a titre of 1 in 100 is the optimal during 2-4 weeks and 1 in 50 during first week**

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Preparation of antigens

The stock culture collection of leptospires is best maintained in screw-capped test tubes containing 5-6 ml of liquid medium. Fresh sub culture are made by inoculating 0.5 ml from the each strain/serovar into tubes. At the same time, a loop-full of the culture should be examined by dark field microscopy to confirm the presence of viable leptospires and the absence of contamination and it should be free from “breeding nests”. The inoculated cultures are incubated at 30°C and checked for the presence of the growth after 5-7 days. The cultures used as antigens should be checked by MAT against homologous antisera frequently for quality control.

Live antigens

The culture may be used directly as the antigen. The antigens can be used up to one week after 5-7 days of incubation.

Formalinized antigens

The live cultures killed by addition of a final 0.5% formaldehyde solution may be used as antigens. These antigens are stable for at least two months. The disadvantage of these antigens is that leptospires tend to adhere loosely to one another, thus interfering with the reading of the reaction.

Density of the antigens

Well grown culture, a minimum density of $1\times10^8$ leptospires per ml should be used as an antigen. The density can be determined by direct counting, using spectrophotometer or by McFarland’s scale.

Procedure

- Fill all 96 wells of microtitre plate with 50 µl PBS.
- Add another 140 µl PBS to the wells of column 2.

Table 1. Commonly used serovars/strains for MAT

<table>
<thead>
<tr>
<th>SEROGROUP</th>
<th>SEROVAR</th>
<th>STRAIN</th>
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<tbody>
<tr>
<td>Andamana</td>
<td>Andamana</td>
<td>CH 11</td>
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<td>Australis</td>
<td>Ballico</td>
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<td></td>
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<td>Jez Bratislava</td>
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</tr>
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<td>RGA</td>
</tr>
<tr>
<td></td>
<td>Copenhageni</td>
<td>M 20</td>
</tr>
<tr>
<td>Javanica</td>
<td>Javanica</td>
<td>Veldrat Bat. 46</td>
</tr>
<tr>
<td></td>
<td>Pol</td>
<td>Poi</td>
</tr>
<tr>
<td>Panama</td>
<td>Panama</td>
<td>CZ 214</td>
</tr>
<tr>
<td>Pomona</td>
<td>Pomona</td>
<td>Pomona</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Pyrogenes</td>
<td>Salinem</td>
</tr>
<tr>
<td>Shermani</td>
<td>Shermani</td>
<td>LT 821</td>
</tr>
<tr>
<td>Sejroe</td>
<td>Sejroe</td>
<td>M 84</td>
</tr>
<tr>
<td></td>
<td>hardjo</td>
<td>Hardjoprajitno</td>
</tr>
<tr>
<td>Semaranga</td>
<td>Patoc</td>
<td>Patoc 1</td>
</tr>
<tr>
<td>Tarassovi</td>
<td>Tarassovi</td>
<td>Perepelitsin</td>
</tr>
</tbody>
</table>
Leptospirosis

- Add 10 µl of serum to the wells of column 2 (dilution is now 1:20) mix and discard 100 µl.
- Dilute by pipetting 50 µl from one well to the next, discard the final 50 µl (2- >3- >4- >5- >6- >7- >8- >9- >10- >11- >12).
- Add 50 µl leptospira culture to all wells.
- Mix thoroughly on a micro shaker.
- Incubate for 2-4 hours at 30ºC.

Reading of the test results
The serum-antigen mixtures are examined under a dark field microscope for agglutination. For observation, one drop of mixture is transferred with a platinum loop or pipette from a well to a microscopic slide and examined under dark field microscope with 20X objective without cover slip.

The titre is the dilution that gives 50% agglutination, leaving 50% of cells free. Compare with a control suspension of leptospires diluted one in two in PBS without serum (column 1).

Notice that with killed antigen observed agglutination is measured directly, contrary to the procedure to the live antigen where, essentially, agglutination is measured indirectly by establishing the reduction of leptospiral density with 50% in comparison with the density of free leptospires in the control.

Note: Serum samples will be stored at -20ºC. Prior to examination, serum samples will be checked for the presence of debris or contamination by bacteria that may interfere with agglutination.

Criteria for serological diagnosis of leptospirosis by using MAT
Seroconversion or four fold rise in antibody titre in paired sera.

A minimum titre of 1:400 or more in a single serum sample. However, the significant titre in the case of single serum samples may vary from one geographical area to other.

Advantages
- It is serovar/serogroup specific test. Some clue about the infecting serovar can be obtained.
- It has sensitivity and excellent specificity.
- Once infected, the person stays MAT Positive for several years. So the test is useful for epidemiological purpose.

Disadvantages
- 14-21 strains have to be maintained in culture, which is often very difficult.
- Procedure is complex and time consuming.
- Reading results requires experienced personnel.
- It is not possible to distinguish between IgM antibodies indicative of current infection, and IgG antibodies indicative of past infection.
- Finding of agglutinating antibodies in a single serum sample does not necessarily prove current leptospirosis. An antibody titre may be due to residual antibodies of a past infection. Therefore, the interpretation of a single titre is not easy so a second serum sample is required for demonstrating a raising titre which has a diagnostic significance.
- Calibration of significant titre is essential in the case of single serum specimens.
- False negativity in the early course of the disease.

Comments: In the early stage of the disease cross reactivity may occur and patient sera may agglutinate a variety of other serovars. Sometimes even to higher titres than the causative serovar, a phenomenon is called as ‘paradoxical reaction’. However, such paradoxical reactions tend to drop in titre and usually the homologous titre to the causative organisms predominate in later stages of the disease.

Other serological methods include Immuno Fluorescent Antibody test (IFA) and Indirect Haemagglutination test (IHA). Different
methods used in the laboratory for the diagnosis of leptospirosis can also be categorized as microscopic, bacteriological, immunological/serological or molecular biological (fig. 14).

**Criteria for a definite diagnosis of Leptospirosis**

The criterion for laboratory diagnosis varies from one laboratory to the other. The criteria usually used in well established laboratories are mentioned below:

- Isolation of leptospires from a clinical specimen.
- Four-fold or greater rise in the MAT titre between acute and convalescent-phase serum specimens run in parallel.
- Sero-conversion from a titre < 1 in 20 to 1 in 80 in between acute and convalescent-phase samples run in parallel.

Isolation of leptospires is laborious and takes several weeks or months. Sero-conversion or rise in titre is the central dogma of serological diagnosis but it requires second convalescent-phase sample which is difficult to obtain. Therefore, the criteria for definite diagnosis has
greater application in establishing the endemicity of the disease in a particular geographical region rather than in routine diagnosis. Once the endemicity is defined in a particular region, criteria for a presumptive diagnosis can be used for diagnosis and case management.

**Criteria for a Presumptive Diagnosis**
- A MAT titre of 100/200/400 or above in single sample based on endemicity.
- A positive result in IgM based immunoassays, slide agglutination test or latex agglutination test.
- Demonstration of leptospires directly or by staining methods.

No single MAT titre can be regarded as diagnostic of acute or active infection and sometimes confirmed patients have low MAT titres. Cross reactive antibodies in syphilis, relapsing fever, lyme disease, enteric fever, dengue and malaria may give a titre of 80 or 100. Therefore low MAT titres need to be interpreted with caution.
References


The taxonomic status of leptospirosa can be defined by a combination of its various characteristics. Therefore different markers should be considered or tests should be repeated. The scheme used for classification and identification of leptospires is shown in Table 1.

Table 1: Scheme used for classification and identification of leptospires

<table>
<thead>
<tr>
<th>Taxonomic Status</th>
<th>Main Characteristic or Test used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Genus</td>
<td>morphology, movement</td>
</tr>
<tr>
<td>2. Species</td>
<td>growth at 13°C and growth with 8-Azaguanine (225 µg/ml),</td>
</tr>
<tr>
<td>3. Serogroup</td>
<td>agglutination with ‘group sera’</td>
</tr>
</tbody>
</table>
| 4. Serovar       | a) agglutination test and agglutinin absorption tests  
b) factor analysis with factor sera. |
| 5. Genomic Species | a) DNA Hybridization  
b) RFLP  
c) RAPD  
d) REA |

Differentiation of pathogenic and saprophytic leptospires

On the basis of morphological characteristics, pathogenic leptospires cannot be differentiated from saprophytic ones. The commonly used tests to discriminate between saprophytic and pathogenic leptospires grow at low temperature (13°C) and resistant to 8-Azaguanine (225 µg/ml). Pathogenic leptospires does not grow at 13°C and they are resistant to 8-Azaguanine.

8-Azaguanine Test

Procedure
- Make suspension of 100 ml distilled water and 225 mg. 8-Azaguanine
- Sterilize the flask with the 8-Azaguanine solution at 121°C for 30 min.
- Add aseptically 0.5 ml 8-Azaguanine solution to 4.5 ml EMJH media
- Mix thoroughly
- Inoculate tubes with 0.05 ml of a well-grown culture of the strain under investigation and the control
- Incubate at 30°C
- Check growth twice in a week up to 21 days

Controls
- Pathogenic strains with and without 8-Azaguanine
- Saprophytic strains with and without 8-Azaguanine

13°C Test

Procedure
- Inoculate 0.05 ml of a well grown culture into 5 ml of EMJH medium of the strain under investigation and the controls in duplicate.
- Incubate one set at 13°C and the other at 30°C
- Check twice in a week by dark field microscopy for growth
- Use controls (Pathogenic strains and Saprophytic strains)
<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Australis</td>
<td>australis</td>
<td>Ballico</td>
<td>Negative</td>
</tr>
<tr>
<td>2. Australis</td>
<td>bratislave</td>
<td>Jez Brat.</td>
<td>Negative</td>
</tr>
<tr>
<td>3. Autumnalis</td>
<td>bangkinang</td>
<td>Bangkinang I</td>
<td>1:80</td>
</tr>
<tr>
<td>4. Autumnalis</td>
<td>butembo</td>
<td>Butembo</td>
<td>Negative</td>
</tr>
<tr>
<td>5. Autumnalis</td>
<td>carlos</td>
<td>3C</td>
<td>Negative</td>
</tr>
<tr>
<td>6. Autumnalis</td>
<td>rachmati</td>
<td>Rachmat</td>
<td>Negative</td>
</tr>
<tr>
<td>7. Ballum</td>
<td>ballum</td>
<td>Mus 127</td>
<td>Negative</td>
</tr>
<tr>
<td>8. Ballum</td>
<td>keny</td>
<td>Njenga</td>
<td>Negative</td>
</tr>
<tr>
<td>9. Bataviae</td>
<td>bataviae</td>
<td>Swart</td>
<td>Negative</td>
</tr>
<tr>
<td>10. Canicola</td>
<td>canicola</td>
<td>H.Uterecht IV</td>
<td>Negative</td>
</tr>
<tr>
<td>11. Canicola</td>
<td>schueffneri</td>
<td>VI.90 C</td>
<td>Negative</td>
</tr>
<tr>
<td>12. Celledoni</td>
<td>celledoni</td>
<td>Celledoni</td>
<td>Negative</td>
</tr>
<tr>
<td>13. Cynopteri</td>
<td>cynopteri</td>
<td>3522 C</td>
<td>Negative</td>
</tr>
<tr>
<td>14. Djasiman</td>
<td>djasiman</td>
<td>Djasinman</td>
<td>1:40</td>
</tr>
<tr>
<td>15. Grippotyphosa</td>
<td>grippotyphosa</td>
<td>Moskva V</td>
<td>1:10240</td>
</tr>
<tr>
<td>16. Grippotyphosa</td>
<td>huanuco</td>
<td>M 4</td>
<td>1:160</td>
</tr>
<tr>
<td>17. Hebdomadis</td>
<td>hebdomadis</td>
<td>Hebdomadis</td>
<td>Negative</td>
</tr>
<tr>
<td>18. Hebdomadis</td>
<td>worsfoldi</td>
<td>Worsfold</td>
<td>Negative</td>
</tr>
<tr>
<td>19. Icterohaem.</td>
<td>copenhageni</td>
<td>M 20</td>
<td>Negative</td>
</tr>
<tr>
<td>20. Icterohaem.</td>
<td>icterohaem</td>
<td>RGA</td>
<td>Negative</td>
</tr>
<tr>
<td>21. Javanica</td>
<td>poi</td>
<td>Poi</td>
<td>Negative</td>
</tr>
<tr>
<td>22. Louisiana</td>
<td>louisiana</td>
<td>LSU 1945</td>
<td>Negative</td>
</tr>
<tr>
<td>23. Manhao</td>
<td>manhao</td>
<td>L 60</td>
<td>Negative</td>
</tr>
<tr>
<td>24. Mini</td>
<td>mini</td>
<td>Sari</td>
<td>Negative</td>
</tr>
<tr>
<td>25. Panama</td>
<td>panama</td>
<td>CZ 214 K</td>
<td>Negative</td>
</tr>
<tr>
<td>26. Pomona</td>
<td>pomona</td>
<td>Pomona</td>
<td>Negative</td>
</tr>
<tr>
<td>27. Pyrogenes</td>
<td>pyrogenes</td>
<td>Salinem</td>
<td>Negative</td>
</tr>
<tr>
<td>28. Sarmin</td>
<td>rio</td>
<td>Rr 5</td>
<td>Negative</td>
</tr>
<tr>
<td>29. Sarmin</td>
<td>weaveri</td>
<td>CZ 390</td>
<td>Negative</td>
</tr>
<tr>
<td>30. Sejroe</td>
<td>hardjo</td>
<td>Hardjopraj</td>
<td>Negative</td>
</tr>
<tr>
<td>31. Sejroe</td>
<td>saxkoebing</td>
<td>Mus 24</td>
<td>Negative</td>
</tr>
<tr>
<td>32. Shermani</td>
<td>shermani</td>
<td>1342 K</td>
<td>Negative</td>
</tr>
<tr>
<td>33. Tarassovi</td>
<td>bakeri</td>
<td>LT 79</td>
<td>Negative</td>
</tr>
<tr>
<td>34. Tarassovi</td>
<td>mogden</td>
<td>Compton</td>
<td>Negative</td>
</tr>
<tr>
<td>35. Tarassovi</td>
<td>rama</td>
<td>316</td>
<td>Negative</td>
</tr>
<tr>
<td>36. Tarassovi</td>
<td>tarassovi</td>
<td>Perepelcin</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Strain DS2 probably belongs to serogroup Grippotyphosa

**DETERMINATION OF SEROGROUP**

Presently there are more than 230 pathogenic serovars, placed into 25 serogroups. To determine group reactivity, the unknown strain X has to be tested in the Microscopic Agglutination Test (MAT) with rabbit antisera “group sera”. A representative group serum is a selected rabbit antiserum, which reacts strongly with all serovars of a certain serogroup but in general gives little cross reactions with strains of other serogroups. Table 2 shows the results obtained in MAT with group sera on an isolate D-15.

**MAT with positive groups**

If the unknown strain (X) reacts positively with one or more group sera the MAT will be
performed with all reference antisera which belong to the positive group(s). If necessary an antiserum against the unknown strain (X) can be produced in a rabbit. The antiserum has to be tested in the MAT with all reference strains which belong to the positive group.

**Calculation of the results**

Cross agglutination titres are expressed as percentages of the reciprocal titre for the homologous strains of positive antisera from the whole serogroup with the unknown strain (X) and vice versa. Two strains are considered to be related in some way if the agglutination titre of unknown strain (X) with an antiserum and/or the agglutination titre of antiserum (X) with reference strain are more than 10%. Titres are expressed using the formula

\[
\frac{T_{gs}}{T_{ui}} = \frac{T_{gi}}{T_{usi}} \times 100
\]

where \(T_{ui}\) is the agglutination titre of the unknown strain, \(T_{gs}\) is the reciprocal titre of antisera from positive group(s) with unknown strain and \(T_{hs}\) is the reciprocal of reference antiserum with homologous strain and

\[
\frac{T_{gi}}{T_{usi}} = \frac{T_{us}}{T_{hi}} \times 100
\]

where \(T_{us}\) is the agglutination titre of unknown sera, \(T_{gi}\) is the reciprocal titre of unknown sera with reference strains from positive groups and \(T_{hi}\) is the reciprocal titre of the unknown serum with homologous strain.

Strain DS-15 and bananal are unrelated as in both sera less than 10% homology is observed. Strain DS-15, canalzona and grippotyphosa are related as in both sera more than 10% homology was observed (Table 3).

**DETERMINATION OF SEROVAR STATUS**

The serovar is the basic taxon in the taxonomy of leptospires. So it is important to characterize an unknown strain of leptospira to the level of the serovar. The methods used for this purpose include the Cross Agglutination Absorption Test (CAAT), the factor sera analysis, typing based on Monoclonal antibodies (serological) and Restriction Enzyme nuclease Analysis (REA), Restriction Fragment Length Polymorphism (RFLP) technique, Random Amplified Polymorphic DNA (RAPD) fingerprinting technique.

**Cross Agglutination and Absorption Test (CAAT)**

Two strains are considered to belong to different serotypes/serovars if after cross-absorption with adequate amounts of heterologous antigen 10% or more of the homologous titre regularly remains in at least one of the two antisera in repeated tests.

The amount of antigen to be used for absorption is a very important factor (Wolff and Broom, 1954; Kmety et al 1970). It is important that the quantitative relationship between antigen and immune serum to be absorbed should be well balanced to avoid the possible non-specific absorption by excessive amounts of antigen (babudieri, 1971). Therefore variable amounts of antigen are mixed with the antiserum. This antiserum is standardized to a MAT titre of 1:5120. The absorbed antiserum whose titre with the absorbing strain is approaching zero (should not exceed 1% of the pre-absorption titre) is used for the agglutination with the homologous strain. Tests to be repeated several times.

All cross agglutination titres of more than 10% are considered to be positive

**Perform absorption test with**

- All relevant reference antisera that react positively with the unknown strain X.
- All relevant reference strains that react positively with the produced antiserum against unknown strain (X).

**The following control test have to be performed prior to absorption test**

- In the cross-absorption test, those pre-dilutions of antisera are used, which give
a MAT titre of 1:5120 with the homologous killed antigen.

- If the MAT titre of the antiserum is higher than 1:5120, dilute to the desired concentration.
- Add 0.4 ml pre-diluted antiserum to 3.6 ml PBS pH 7.2 with formalin (0.5%).
- 3 ml of this diluted antiserum is used for the absorption tests with different amounts of sedimented leptospires and 1 ml is used for the homologous control test.

### Inoculation and centrifugation

- Inoculate a well-grown culture into 50 ml EMJH medium.
- Incubate in the shaking incubator for 5-7 days at 30°C.
- Check the culture by dark field examination for the absence of debris and contamination.
- Treat the well-grown cultures (minimum density = 2 x 10^8 leptospires/ml) with formalin (0.5% final concentration).
- The formalinized cultures are allowed to stand for one hour.
- Divide the culture over 5, 10 and 20 ml leptospira quantities and centrifuge the three different amounts of leptospira culture for 30 min. at 10,000 g.
- After centrifugation the supernatants have to be discarded.

### Absorption

- Re-suspend the sediments with air-dried cells of strain X (derived from 5, 10 and 20 ml culture) in 1 ml PBS-formalin diluted antiserum each.
- Absorption of the antiserum with the sediments has to be performed overnight at 30°C.
- Spin down the leptospires by centrifugation for 30 minutes at 10,000 g.
- The supernatants are pipetted off carefully.
- The supernatants are used for the MAT.

### Agglutination after absorption

- To check for over or under absorption, the absorbed reference antisera are tested in the MAT with live and killed antigen (absorbing strain X).
- Fill all the wells of microtitre plate with 50 µl of PBS pH 7.2.
- Add 50 µl each of the absorbed antiserum to be tested to the wells of column 2. The dilution in this well is now 1:20.
- Add to all wells 50 µl antigen (absorbing stain X). After the addition of the antigen the dilution will be 1:40 in column 2.
- After reading the MAT one has to chose on from the three absorbed antiserum for further testing. Take the absorbed antiserum each having a MAT titre of 1:40 or 1:80. This titre has to be less than 1% of the homologous titre for the same antiserum.

### Table 3: Cross agglutination absorption test results on isolate DS-15

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Abs. Strain</th>
<th>Homologous Titre before absorption</th>
<th>Homologous Titre after absorption</th>
<th>Relation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L 5120 K 5120</td>
<td>L 5120 K 5120</td>
<td>100</td>
</tr>
<tr>
<td>Canalzonae</td>
<td>DS-15</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>DS-15</td>
<td>Canalzonae</td>
<td>L 10240 K 5120</td>
<td>L 5120 K 2560</td>
<td>50</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>DS-15</td>
<td>L 10240 K 1520</td>
<td>L 320 K 160</td>
<td>3.1</td>
</tr>
<tr>
<td>DS-15</td>
<td>Grippotyphosa</td>
<td>L 2560 K 2560</td>
<td>L 40 K 80</td>
<td>6.2</td>
</tr>
</tbody>
</table>
With the chosen absorbed antiserum the following MATs are performed

- Control test of the unabsorbed diluted reference antiserum with live and killed homologous reference antigen.
- Test of the absorbed serum with live and killed homologous reference antigen.
- The tests can also be performed with the produced antiserum against strain X with all positive reference strains.

Calculation of the results

Interpretation of results

*Relationship between strain DS-15 and canalzona:* In both sera more than 10% of the homologous titre remains. They belong to the different serovars.

*Relationship between strain DS-15 and grippotyphosa:* Both strains are related as after absorption both the serum samples give less than 10% homologous titre.

**Typing using monoclonal antibodies (mAbs)**

Mouse monoclonal antibodies (mAbs) are used to type isolates as belong to certain serovars. Characterization can be done by the use of MCAs that react with a single characteristic epitope or with several MCAs that react with a characteristic mosaic of epitopes.

**Histograms**

Serovars can be identified by their characteristic antigenic pattern recognized by a set of monoclonal antibodies which are called histograms.

---

**Equipment**

- Disposable microtitre plates (96 wells)
- Pipettes
- Plastic tips
- Incubator 30°C
- Multichannel diluter
- Dark field microscope (objectives 16-20 X, eye pieces 10 X)
- Microscopic slides

**Reagents**

- Phosphate Buffered Saline (PBS), pH 7.2
- Leptospira reference strains and isolates
- Mouse monoclonal antisera
- Leptospires of control reference strains and isolates will be grown in EMJH medium, incubated for 5-7 days at 30°C and checked by dark field microscopy for adequate density and absence of debris or contaminating bacteria.
References


Recently, DNA-based techniques have been proposed as alternative methods of diagnosis and identification of leptospires. Nucleic acid probes and hybridization techniques were used previously for detection of leptospires from clinical samples\(^1\,^2\). Polymerase Chain Reaction (PCR) was subsequently developed for detection of leptospires from clinical samples. Different groups developed different primers targeting different locations of the leptospiral genome for sequence-specific amplification of sequences in PCR that holds promise as a molecular tool for diagnosis of the disease\(^3\,^4\,^5\). PCR is very often used as a very sensitive and specific test for diagnosis of many diseases and PCR with different primers specific for *Leptospira* is in the process of evaluation in different laboratories across the world.

**Hybridization with Nucleic acid probes**

Nucleic acid hybridization can confirm diagnosis before the results of culture and biochemical tests are available. Dot blot hybridization method with \(^32\)P- and biotin-labeled genotype-specific probes has been used for early detection of leptospires\(^6\). *In situ* hybridization method using biotin-labelled DNA probe for detection of *L. interrogans* in clinical samples has also been proposed. In a particular study, it was found that nucleic acid hybridization detected 60 of 75 urine samples, whereas FAT detected 24 samples and only 13 samples were detected by bacteriological culture. However, hybridization with nucleic acid probes is tedious and costly and with polymerase chain reaction gaining more popularity, the use of labeled probes for molecular diagnostics has diminished.

**Polymerase Chain Reaction (PCR)**

PCR method involves *in vitro* amplification of genus-specific target DNA sequence, if present, in clinical samples. A pair of short DNA fragments, known as primers is used for specific amplification of DNA fragments from the pathogen in blood, urine or CSF. Positive diagnosis results from the amplification of the target sequence whereas negative samples fail to produce amplified DNA in PCR. PCR can be used to detect leptospiral infection in both animals and human beings following the same methods. Van Eys et al\(^7\) developed PCR for detection of leptospires in urine samples of infected cattle. Urine samples containing as few as 10 leptospires per ml gave positive results in PCR assay. Gravekamp et al\(^4\) proposed the use of two sets of primers (G1 & G2 and B641 & B651) that enabled the amplification of target DNA fragment from leptospiral species. Amplification results in generation of a PCR product of 285 bp and could detect even 1-10 leptospires per ml of urine. Leptospires were also detected from aqueous humor of a patient with unilateral uveitis using silica particles and guanidine thiocyanate method by PCR\(^8\). It has been found that PCR is a rapid, sensitive and specific means of diagnosing leptospiral infection, especially during the first few days of the disease when antibodies are not fully detectable in serological tests. In different studies involving patients suffering clinically with acute leptospirosis, PCR has been compared with bacterial culture and MAT for diagnosis and it has been concluded that the PCR is an
efficient tool for early diagnosis of leptospirosis during acute phase of the disease, especially when the clinical symptoms are confusing. The most important advantages of PCR are its sensitivity, specificity and rapidity through which the disease can be diagnosed. However, the major drawbacks of this technique are its high operational cost and unavailability of facilities in common diagnostic laboratories.

The detection of leptospires in the clinical samples by using PCR is one of the most valuable additions in the laboratory for diagnosis of leptospirosis. PCR is an iterative process consisting of three elements:

1. Denaturation of the template DNA into two separate strands at an optimal temperature and time depending on their G+C content and size of the template DNA.

2. Annealing of the oligonucleotide primers to the single stranded sequences at an optimal temperature of 3-5ºC lower than the calculated melting temperature at which the oligonucleotide primers dissociate from their templates.

3. Extension of the annealed primers with the help of dNTPs and catalyzed by the thermostable DNA polymerase. However, number of cycles required for amplification of DNA depends on the number of copies of template DNA present at the time of beginning of the reaction and the efficiency of primer extension and amplification. Once the reaction starts it proceeds until one of the components becomes limiting.

**Methods**

**Serum or Plasma preparation for PCR**
- Mix serum sample with L6 lysis buffer [120 g of GuSCN, 22 ml of 0.2 M EDTA, 2.6 ml of Triton X 100 in 100 ml of 0.1 M Tris Hcl (pH 6.4) in the ratio of 1:9 (V/V)].
- Add 40 μl of diatom suspension [0.5 ml of 36% (W/V) HCl and 10 g of diatoms (Kieselguhr-DG) in 50 ml of H2O].
- Incubate the mixture after vortexing thoroughly at room temperature for 10 min.
- Centrifuge the mixture for 2 min. at 16,000 g and sediment the diatoms.
- Wash the diatoms with absorbed DNA twice with 1 ml of L2 wash buffer [120 g of GuSCN in 100 ml of 0.1 M Tris Hcl (pH 6.4)], twice with 1 ml of 70% (V/V) ethanol and once with 1 ml of acetone.
- Dry the diatoms pellets at 56ºC.
- Elute the DNA in 120 μl of double distilled H2O for 10 min at 56ºC in presence of 5 μl of 10 mg/ml Proteinase K.
- Inactivate the Proteinase K by incubating the sample at 100ºC for 15 min.
- Centrifuge for 2 min at 16,000 g.
- Use 10 to 40 μl of eluted DNA sample for PCR.

**Urine Sample Preparation for PCR**
- Collect 10 ml of Urine in a 15 ml container and add 10 μl formalin (final concentration 0.1%) and store the sample at 4ºC till use.

**Materials and Equipment**
1. Tris
2. Guanidinethiocyanate
3. EDTA
4. Triton x 100
5. Sodium hydroxide
6. Kieselguhr-DG
7. Proteinase K
8. Ethidium bromide
9. Taq DNA Polymerase
10. Assay buffer for Taq DNA Polymerase
11. dNTPs mixture
12. Sets of primers (specific for Leptospira)
13. Mineral oil

**Equipment**
1. PCR thermo cycler
2. Submarine horizontal gel electrophoresis apparatus
3. UV transilluminator
4. Vorter mixer
5. Water bath shaker
6. Micro centrifuge
7. Eppendorf micro centrifuge tube
8. PCR tube
9. Micropipettes and mcrotips
10. Camera and film
Centrifuge 10 ml of Urine for 30 min at 1500 g.
Discard the supernatant.
Add 9 ml of L6 lysis buffer and 40 μl of diatoms or silica to the pellet.
Incubate by shaking for 10 min at RT.
Centrifuge for 5 min at 1500 g.
Discard supernatant by suction.
Add 1 ml of L2 wash buffer and transfer the suspension to a clean 1.5 ml tube.
Wash the pellets twice with 1 ml L2 wash buffer (1 min at 16000 g).
Wash the pellets twice with 1 ml 70% ethanol (1 min at 16000 g).
Wash the pellets once with 1 ml acetone (1 min 16000 g).
Dry the pellets for 10 min at 56ºC.
Elute the DNA in 120 μl of double distilled H₂O for 10 min. at 56ºC in presence of 5 μl of 10 mg/ml Proteinase K.
Boil the eluted DNA solution for 10 min at 100ºC.
Centrifuge for two min at 16,000 g.
Collect 100 μl supernatant with a pipette and use 10 to 40 μl for the PCR reaction.

**Amplification of DNA**

- The amplification of DNA is performed in a total volume of 50 μl.
- The primers used for the PCR are G1 5' - CTG AAT CGC TGT ATA AAA GT-3' & G2 5' - GGA AAA CAA ATG GTC GGA AG-3' and B64I 5' - CTG AAT TCT CAT CTC AAC TC-3' & B64II 5' - GCA GAA ATC AGA TGG ACG AT-3'
- The reaction mixture (50 μl) contains 5 μl of 10x assay buffer [10 mM Tris HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl and 0.01% Gelatin], 200 μM each dNTPs, 20 pM of each primer, 0.5 U of Taq DNA Polymerase, Template DNA (10-40 μl). Add 20 μl of mineral oil on top of the reaction mixture, if required, to prevent evaporation.
- Amplification is carried out in a thermal cycler for 30 cycles. Each cycle consists of denaturation of DNA at 94ºC for 1 min, annealing of the primer at 55ºC for 1 - 2 min and extension for two min. at 72ºC. After the cycles are completed, the final extension of the amplified product is done at 72ºC for seven min.
- The amplified PCR products are analysed in 1%-2% agarose gel with 0.1 μg/ml ethidium bromide in TAE buffer pH 8.0. At the end of the electrophoresis the gel is visualized under UV transilluminator and photographed. Amplification of 285/563base pair DNA fragment indicates presence of leptospiral DNA in the specimen.

**Advantages**

- Gives relatively quick results in the early stage of the disease when antibodies have not yet developed in detectable levels.

**Disadvantages**

- Complicated and expensive
- Sophisticated equipment are required.
Conventionally leptospires are classified serologically on antigenic properties with a serovar as the basic taxon. However, the Microscopic Agglutination Test (MAT), Cross Agglutination Absorption Test (CAAT) and monoclonal antibody (mAbs) techniques that are used for serological characterizations are generally laborious, time-consuming, and often cannot distinguish all serovars. Moreover, tests like the MAT and CAAT require ready supply of live antigens.

With the advent of modern molecular biological tools, several attempts have recently been made to characterize leptospires by these techniques. Most of these techniques are DNA-based. DNA-based characterization scheme is based on the genetic make up and can translate the genetic code into visible patterns. These methods are highly reproducible and can show true genetic affinities and relationships between isolates.

Some of the methods that are currently being used in characterization of leptospires are:

- Arbitrarily primed PCR (AP-PCR) or Randomly Amplified Polymorphic DNA (RAPD) fingerprinting.
- Restriction Endonuclease Analysis (REA).
- Restriction Fragment Length Polymorphism (RFLP).

**Materials and equipment required**

<table>
<thead>
<tr>
<th>Material/Equipment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8, 10 mM</td>
<td>Magnesium chloride, 4 mM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>KCl 50 mM</td>
</tr>
<tr>
<td>Thermal cycler</td>
<td>Tris EDTA buffer</td>
</tr>
<tr>
<td>Electrophoresis apparatus</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>UV Transilluminator</td>
<td>Agarose type II</td>
</tr>
<tr>
<td>Vortex mixture</td>
<td>RAPD primers</td>
</tr>
<tr>
<td>Water bath shaker</td>
<td>dNTPs</td>
</tr>
<tr>
<td>Micropipettes</td>
<td>Gel documentation system</td>
</tr>
<tr>
<td>Microtips</td>
<td></td>
</tr>
</tbody>
</table>

**Principle**

The AP-PCR, also called random amplified polymorphic DNA fingerprinting technique is based on the amplification of the DNA in the PCR by short random oligonucleotide primers. The size and number of the amplified products depend on the particular primers and template DNA. It is used to compare intra and interspecific differences among pathogens including leptospires. The RAPD technique can be applied on purified DNA, crude extracts of cells from cultures or from colonies on agar plates. This technique is being used for the molecular differentiation/typing of various leptospiral isolates/strains.

**Procedure**

DNA for AP-PCR is prepared following the method of Boom et al. AP-PCR fingerprinting technique is carried out as per the method described by Roy et al. M16, a 22-mer oligonucleotide (5’ AAA GAA GGA CTC AGC GAC TGC G 3’) previously used as one of a pair of primers for amplification of an insertion sequence in Leptospira genome, is used as a primer for AP-PCR, because several regions of Leptospira genome are known to possess base sequences complementary to it.
PCR is performed in 50 ml reaction volumes with 50 ng purified DNA, 2 mM primer, 250 mM of each dNTP, 1.5 mM MgCl2, 0.5 U of Taq polymerase in 10 mM Tris-HCl (pH 9.0) and 50 mM KCl. The temperature programme consisted of 1 cycle of 3 min at 94°C, 1 min at 60°C, and 2 min at 72°C; followed by 38 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C; and a final cycle of 1 min at 94°C, 1 min at 60°C, and 9 min at 72°C. All reaction products are electrophoresed on 20 cm long 1% agarose gels, stained with 0.5 mg/ml ethidium bromide, and photographed under ultraviolet light using gel documentation system. DNA profiles of individual lanes are matched with each other and dendrograms of genetic distance based on similarity-coefficient are generated at 5% confidence level using included software.

RESTRICTION ENDONUCLEASE ANALYSIS (REA)

Principle
The Restriction Endonuclease Analysis (REA) is based upon the fact that DNA of different nucleotide sequences, those which have a different genetic origin will give different band pattern in gel electrophoresis after enzymatic treatment. The REA involves the extraction of DNA from a homogeneous population of organisms, digestion of the DNA with the restriction endonuclease and electrophoresis of the digested DNA in an agarose gel. The restriction endonuclease recognize and cleave double stranded DNA at a specific sequence, hence a set of fragments are generated. These fragments are highly specific for each type of leptospires for their molecular characterization. The REA technique is sensitive enough to differentiate between different leptospiral strains on the basis of genetic differences. By comparing restriction map of different strains, it is possible to differentiate them at molecular level. The technique is very much useful for the molecular identification/typing of leptospiral isolates/strains from various sources.

Preparation of buffers
Tris-borate buffer (TBE buffer) pH. 8.2 stock solution (10 x)
- Tris: 108.0 gm
- Boric acid: 55.0 gm
- EDTA, disodium: 9.3 gm
To one litre triple distilled water

Working solution 1 x TBE buffer
Stock solution of TBE buffer 100 ml is mixed properly with 900 ml of triple distilled water to prepare the working solution of 1 x TBE buffer.

Gel loading buffer
- Bromophenol blue: 0.25 gm
- Xylene cyanol: 0.25 gm
- Sucrose: 40.00 gm
- Single strength Tris borate buffer to: 100 ml

Procedure
The REA is carried out as per the method of Marshall et al\textsuperscript{12}. The restriction digestion is carried out in 20 µl of volume in a sterile microfuge tube, the reaction mixture contains 5 µl of DNA, 10 x RE buffer 2 µl, 10 to 15 units restriction enzymes and triple distilled water to a final volume of 20 µl.

The digestion mixture is incubated in a water bath-shaker at 37°C for the required period of time. After the incubation the reaction mixture (sample) is inactivated by heating at 65°C for 5 min before electrophoresis. For electrophoresis 0.7% agarose gel in TBE buffer containing 5 µg/ml ethidium bromide is used.
Each digested DNA samples are mixed with 1/5th volume of the gel loading buffer and loaded in the wells of agarose gel. Lambda DNA digested with either Pst I, EcoRI, or Hind III are used as DNA molecular weight markers.

Electrophoresis is carried out in a sub-marine electrophoresis apparatus at 5 v/cm at room temperature for 5 to 6 hours, depending upon the length of the gel.

At the end of the electrophoresis the gel is visualized under UV transilluminator and photograph is taken using gel documentation system.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Principle
The Restriction Fragment Length Polymorphism (RFLP) technique is a DNA based technique which was introduced in 1990 as a powerful tool for detecting differences among the chromosomes of organisms that are very closely related as determined by the conventional genomic analysis. By RFLP analysis the difference in DNA sequences are determined by examining the size of the fragments that result when DNA is cleaved with restriction enzymes. RFLP analysis involves the extraction of DNA from a homogeneous population of organisms, digestion of the DNA with the restriction enzymes and electrophoresis of the digested DNA in an agarose gel, southern blotting on to nitrocellulose/nylon membranes and hybridization with specific probes.

The different restriction enzymes have different recognition sites and cleave the DNA at a specific sequence, which produce different banding patterns called as DNA fingerprints. It is now feasible to characterize strains of leptospires/organisms at the genomic level by the unique restriction fragment length polymorphism patterns of their DNA. Therefore this technique is very much useful for the molecular identification of leptospiral isolates/strains from various sources and for epidemiological investigations in the spread of particular strains in the community.

Preparation of buffers
Tris-borate buffer (TBE buffer) pH. 8.2 stock solution (10 x)
Tris 108.0 gm
Boric acid 55.0 gm
EDTA, disodium 9.3 gm
Triple distilled water to 1.0 litre

Working solution 1 x TBE buffer
Stock solution of TBE buffer 100 ml is mixed with 900 ml of triple distilled water to prepare the working solution of 1 x TBE buffer.

Gel loading buffer
Bromophenol blue 0.25 gm
Xylene cyanol 0.25 gm
Sucrose 40.00 gm
Single strength Tris borate buffer to 100 ml

20 X SSC (pH 7.0)
Sodium chloride (3.0 M) 175.32 gm
Trisodium citrate (0.3 M) 88.20 gm
to 1 litre distilled water
5. 0.25 N HCl
20.8 ml of conc. Hcl diluted to a litre with distilled water

Denaturation solution
NaCl (1.5 M) 87.76 gm
NaOH (0.5 M) 20.09 gm
to 1 litre distilled water stored at room temperature

Neutralization solution (pH 7.2)
NaCl (1.5 M) 87.76 gm
Tris - HCl (0.5 M) 60.55 gm
to 1 litre distilled water

Prehybridization and hybridization buffer
Ficoll 400 0.5 gm
Bovine serum albumin 0.5 gm
Polyvinyl pyrralidone 0.5 gm
Sodium dodecyl sulphate 2.5 gm
20X SSC 125 ml
1M Tris/HCl (pH 7.5) 25 ml
0.5M EDTA 1 ml
Deionized formamide 250 ml

Make the volume up to 500 ml with sterilized triple distilled water.

**Procedure**

The restriction digestion is carried out in 20 µl of volume in a sterile microfuge tube, the reaction mixture contains 10 µl of DNA, 10 X RE buffer 4 µl, 3 µl restriction enzyme and triple distilled water to a final volume of 20 µl.

The reaction mixture is incubated in a water bath shaker at 37ºC overnight for complete digestion. After incubation the reaction mixture (sample) is inactivated by heating at 65ºC for five min before electrophoresis. For electrophoresis 1% to 2% agarose gel in TBE buffer containing 5 µg/ml ethidium bromide is used.

Each digested DNA sample is mixed with 1/5th volume of the gel loading buffer and loaded in the agarose gel. Lambda DNA digested with either EcoRI, Hind III or Pst I can be used as a DNA molecular weight markers.

Electrophoresis is carried out in a submarine electrophoresis apparatus at 5 V/cm at room temperature for 5 to 6 hours or 3 V/cm overnight. At the end of the electrophoresis the gel is visualized under UV transilluminator to see the positions of the bands, after that acidic, alkaline and neutralization treatment is given to the gel before transfer onto nitrocellulose/nylon membrane, to keep the DNA strands separate.

Southern blotting is done onto nitrocellulose membrane using transfer buffer for 20-24 hours as described by Southern (1975). After blotting the membrane is dried at room temperature and baked at 80ºC for two hours in a vacuum oven to fix the DNA to the nitrocellulose membrane.

The nitrocellulose membrane is placed in a hybridization tube with hybridization buffer (10

### Materials and equipment required

<table>
<thead>
<tr>
<th>Material/Equipment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris borate buffer (pH 8.2)</td>
<td>20 X SSC (pH 7)</td>
</tr>
<tr>
<td>Sodium saline citrate</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>EDTA</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Agarose type II</td>
</tr>
<tr>
<td>Denaturation solution</td>
<td>Neutralization solution</td>
</tr>
<tr>
<td>Hybridization solution</td>
<td>Triple distilled water</td>
</tr>
<tr>
<td>Restriction enzymes with appropriate buffers</td>
<td>Lambda DNA marker</td>
</tr>
<tr>
<td>y-p or DNG labelling kit for specific probe labeling &amp; ECL detection kit</td>
<td>Random primed labeling kit</td>
</tr>
<tr>
<td>Nitrocellulose/Nylon membranes</td>
<td>Whatman’s No. 3 filter paper</td>
</tr>
<tr>
<td>Probe DNA can either be DNA</td>
<td>rRNA</td>
</tr>
<tr>
<td>a PCR product eluted from the gel</td>
<td>a synthetic oligonucleotide having complementary sequence to leptospiral DNA</td>
</tr>
<tr>
<td>Electrophoresis apparatus</td>
<td>UV Transilluminator</td>
</tr>
<tr>
<td>Water bath shaker</td>
<td>magneti stirrer</td>
</tr>
<tr>
<td>Micropipettes &amp; Microtips</td>
<td>Hybridization oven</td>
</tr>
<tr>
<td>-20 ºC and -70 ºC</td>
<td>Vacuum blotter</td>
</tr>
<tr>
<td>Vortex mixture</td>
<td>Gel casting platform with comb</td>
</tr>
<tr>
<td>X-ray film &amp; Auto-radiography cassettes with intensifying screens</td>
<td></td>
</tr>
</tbody>
</table>

---

Leptospiriosis
to 15 ml) for pre-hybridization at 60°C for two hours. Then specific probes (DNA, rRNA, cDNA & synthetic oligonucleotide having complementary sequence to leptospiral DNA) labelled either by P³² or with digoxigenin, ECL based non-radioactive method is added to the pre-hybridized membrane in hybridization solution.

The hybridization is carried out and incubated at 60°C for 18 to 24 hours. After hybridization the nitrocellulose membrane is washed twice for 15 min in 2 X SSC, once for 15 min in 0.1 X SSC at 50°C. After washing the membrane is dried at room temperature and exposed overnight to X-ray film in an auto-radiographic cassettes with intensifying screen at -70°C in the case of radioactive probe. After enough exposure the X-ray film is removed from the cassette and developed.

In the case of non-radioactive labeling detection system, the membrane is directly treated with ECL luminescent detection buffer/system. If the samples have complementary nucleic acid sequences to the probe, the strong bands of restriction fragments are seen/detected on the X-ray film/Nitrocellulose membrane within 15-30 min.
References

Preparation of EMJH Medium

ANNEXURE I

Glassware
Conical flasks
Measuring cylinders
Pipettes

<table>
<thead>
<tr>
<th>Chemicals required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
</tr>
<tr>
<td>Thiamine</td>
</tr>
<tr>
<td>Bovine Serum Albumin Fraction V</td>
</tr>
<tr>
<td>CaCl$_2$ 2H$_2$O</td>
</tr>
<tr>
<td>MgCl$_2$ 6H$_2$O</td>
</tr>
<tr>
<td>FeSO$_4$ 7H$_2$O</td>
</tr>
<tr>
<td>CuSO$_4$ 5H$_2$O</td>
</tr>
<tr>
<td>ZnSO$_4$ 7H$_2$O</td>
</tr>
<tr>
<td>Tween 80</td>
</tr>
</tbody>
</table>

Stock solutions

Albumin fatty acid supplement stock solution

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams per 100 ml H$_2$O</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ 2H$_2$O + MgCl$_2$ 6H$_2$O</td>
<td>1.0 each</td>
<td>- 20°C</td>
</tr>
<tr>
<td>ZnSO$_4$ 7H$_2$O</td>
<td>0.4</td>
<td>- 20°C</td>
</tr>
<tr>
<td>CuSO$_4$ 5H$_2$O</td>
<td>0.3</td>
<td>+ 4°C</td>
</tr>
<tr>
<td>Vitamin B$_{12}$</td>
<td>0.02</td>
<td>- 20°C</td>
</tr>
<tr>
<td>Tween 80</td>
<td>10</td>
<td>- 20°C</td>
</tr>
</tbody>
</table>

Basal medium stock solution

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams per 100 ml H$_2$O</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4$Cl</td>
<td>25.0</td>
<td>- 20°C</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.5</td>
<td>- 20°C</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10</td>
<td>- 20°C</td>
</tr>
</tbody>
</table>

Method of Preparation

Albumin Fatty Acid Supplement
- Dissolve 10 gms of bovine serum albumin in 60 ml. sterile glass distilled water.

- Take necessary stock solutions out of the freezer.
- Add 1.5 ml Calcium Chloride and Magnesium Chloride stock solution.
- Add 1 ml Zinc Sulphate stock solution.
- Add 100 µl Copper Sulphate stock solution.
- Add 50 mg Ferrous Sulphate and 40 mg Sodium Pyruvate.
- Add 1 ml of Vit.B12 Stock solution.
- Add 12.5 ml Tween 80 stock solution.
- Add 24 ml Sterile distilled water to make up final volume of 100 ml.
- Adjust pH to 7.4 - 7.6 with 1 N NaOH.
- Filter through using a Millipore filter pore size 0.22 µm.

Basal Medium
- Dissolve 1 gm of Na$_2$HPO$_4$ and 300 mg of KH$_2$PO$_4$ and 1 gm of NaCl in 100 ml glass distilled water.
- Add 1 ml Ammonium Chloride stock solution.
- Add 1 ml Thiamine stock solution.
- Add 1 ml Glycerol stock solution.
- Add glass distilled water to make up a total vol. of 900 ml.
- Adjust pH to 7.4.
- Finally make the volume to 1000 ml by adding glass distilled water.
- Autoclave at 121°C for 30 min.

Preparation of Complete Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Parts</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Medium</td>
<td>9</td>
<td>900 ml</td>
</tr>
<tr>
<td>Albumin Fatty Acid Supplement</td>
<td>1</td>
<td>100 ml</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Preparation of Fletcher’s Medium

ANNEXURE II

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Stock Solutions</th>
<th>Method of Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>Sorensen Buffer A: Dissolve 11.876 gms. Na₂HPO₄ in one litre of distilled water.</td>
<td>1. Add 80.8 ml. Sorensen Buffer A and 19.2 ml Sorensen Buffer B.</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Sorensen Buffer B: Dissolve 9.078 gms. KH₂PO₄ in one litre distilled water.</td>
<td>2. Mix thoroughly.</td>
</tr>
<tr>
<td>NaCl</td>
<td>Both Buffer solutions are autoclaved at 121°C for 30 min and stored at 4°C.</td>
<td>3. Adjust the pH to 7.4 - 7.6.</td>
</tr>
<tr>
<td>Bacto peptone</td>
<td>Stock Solutions</td>
<td>4. Heat until all ingredients are dissolved.</td>
</tr>
<tr>
<td>Bacto Beef Extract</td>
<td></td>
<td>5. Dispense the medium in bottles, 184 ml per bottle.</td>
</tr>
<tr>
<td>Agar</td>
<td></td>
<td>6. Autoclave all the bottles at 121°C for 30 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7. Store at 4°C.</td>
</tr>
</tbody>
</table>

Preparation of final medium
1. Collect rabbit serum with a little bit of red blood cells.
2. Inactivate the serum for 30 min at 56°C in a water bath.
3. Add 16 ml of serum to 184 ml medium.
4. Dispense the medium in 4 to 5 ml volume in screw capped test tubes.
5. Check for sterility by incubating the tube for 48 hrs at 37°C.
6. Store the medium at 4°C until use.
Routine cultures
Stains used routinely as sources of antigens for serological tests or other purposes are maintained in liquid medium dispensed in 5 ml amounts in screw-capped test tubes. Duplicate tubes should be inoculated. The inoculum should be approximately 10% of the culture volume and should be examined microscopically to confirm the presence of viable organisms and the absence of contamination. Cultures are kept at room temperature after incubation of 5 to 7 days at 30°C. Cultures are routinely transferred at 2 to 3 week’s intervals.

Stock Culture
Stock cultures are best maintained in tubes of semi-solid media and stored in dark at room temperature. Cultures are viable for at least three months and frequently as long as one year. The process of sub-culture is the same as above.

Leptospires cannot readily be stored in frozen dried state but may be stored for years in liquid nitrogen by using either glycerol (5 to 10%) or di-methyl sulfoxide (5%) as a cryoprotectant.
**Filtration**
Leptospires can pass through a cellulose membrane filter with pore diameter of 0.22 µm. The contaminated fluid culture may be diluted and filtered through a suitable filter and can be cultured from the filtrate.

**Selective media**
Lightly contaminated cultures may be sub-cultured into a media containing 5-fluro-uracil (100 µg/ml).

<table>
<thead>
<tr>
<th>Plating on solid media</th>
<th>Animal inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptospiral colonies spread horizontally through media containing 1% of agarose, away from the immobile colonies of the contaminating bacteria.</td>
<td>Inject the contaminated culture in a hamster or a guinea pig intra-peritoneally. Take blood by heart puncture after 10 to 15 min and inoculate the blood in culture medium.</td>
</tr>
</tbody>
</table>
Preparation of Antisera

**Selection of Animals**
Young healthy rabbits weighing approximately 3 to 5 kgs should be selected.

The rabbits should be pre-tested for leptospira antibodies prior to inoculation.

**Preparation of Antigens**
Isolates or reference strains should be cloned by culturing on solid media containing 1% agarose.

The antigen is prepared by inoculating a single colony into EMJH liquid medium.

**Procedure**
- Rabbits should be inoculated intravenously into marginal ear veins with successive doses of 5 to 7 days old live culture containing approximately $2 \times 10^8$ leptospires/ml.
- Five injections of 1 ml, 2 ml, 4 ml, 6 ml and a further 6 ml should be given at seven days interval.
- 14 days after the last injection, a small sample of blood should be taken from the ear vein for testing.
- If the homologous titre of serum is between 1:10,000 or above the rabbit should be bled by heart puncture.
- If the titre of the serum is below 1:10,000, a further booster injection of 6 ml of live culture is given.
- At least two rabbits should be used for the preparation of each antiserum.

**Storage of sera**
- Dispense the sera in small amounts, into the sterile ampoules. Lyophilize and store at 4°C.
- Dispense the sera in small amounts (e.g. 2 ml) into the sterile ampoules and store at -70°C or -20°C.
- Treat with preservative and store at 4°C (thiomersal 0.02% or phenol 0.3%).
Requirements for a Leptospirosis Laboratory

Given below are the minimum basic requirements for establishing a small leptospirosis laboratory capable of performing screening and confirmatory tests for the diagnosis of leptospirosis.

Culture media
The common culture media used are Korthof’s medium, Fletcher’s medium and EMJH medium.

Staff and skills required
Special laboratory facilities and skills are required. Leptospires require a lot of care. Cultures need to be sub-cultured at regular intervals and filtered into fresh media whenever necessary. One needs to be well trained or has to employ well trained technicians, who should be solely working on leptospirosis.

Equipment
- Microscope: A good microscope with dark field illumination is essential
- Hot air oven and Autoclave
- Centrifuge
- Micro pipettes
- Balance
- Refrigerator, freezer
- Glass distillation plant
- Laminar flow cabinet

Glass Ware & Plastic Ware
- Screw capped test tubes (125 x 15)
- Conical flasks
- Pipettes
- Pasture pipettes
- Microscopic slides
- Racks for test tubes
The density of leptospira culture can be determined by:

1. **Estimating the number of leptospires per field by dark field microscopy**
   - Grow the leptospira in a liquid media.
   - Add formalin to the culture (final concentration 0.5%).
   - Dilute the culture in a suitable dilution.
   - Transfer 10 μl of diluted culture on a microscopic slide.
   - Apply cover slip.
   - Count the leptospires under dark field microscope.
   - Cultures with a minimum density of 600 - 800 leptospires per field may be used as antigens.

2. **Counting of leptospires using counting chamber (Hawksey or Burker - Truck).**

3. **Measure of the Optical density**
   - Measure the OD of formolized antigens by spectrophotometer using EMJH medium as blank at 420 nm.
   - Culture OD range between 0.052 to 0.1 (Approximately 2-3 X10⁸ leptospires per ml) may be used as an antigen in MAT.

**Using the McFarland scale**
- The culture density corresponding to that of McFarland tube No. 1 (Approximately 2-3 x 10⁸ leptospires)
General Safety Rules for Leptospirosis Laboratories

1. In connection with the dangers of contamination, it is not permitted to smoke, eat or drink in laboratories, with the exception of specially allocated places.

2. All procedures, which could involve chances of direct or indirect contact with the organism or clinical material, are very hazardous. The use of gloves and the wearing of lab coat are thus highly recommended.

3. Hypodermic needles, scalpels, microscopic slides and similar sharp objects should be deposited immediately after use in the allocated containers containing disinfectants. In order to reduce the chance of prick accident, hypodermic needles should never be replaced in the protective sleeves.

4. All the test tubes and vials containing organism or patient material should be thoroughly closed and should be deposited immediately in the allocated containers containing disinfectants and should be autoclaved.

5. When performing procedures whereby aerosol formation, splashing or powder formation can occur, it is mandatory to use protective devices such as safety goggles, face masks, gloves and extractor hoods.

Safety Recommendations

1. Keep the laboratory area and administration as far apart as possible

2. Make sure the work place is orderly with an easily cleanable surface. Keep the benches and work places as empty as possible. Do not sit on worktops. Workplaces and floors must be kept tidy.

3. Pipetting must never be done with mouth. Use an automatic pipette or a pipette using suction-balloon.

4. A clean, disinfected workplace is extremely important in the prevention of contamination. Make sure to wash your hands with soap and water, not only when leaving the workplace, but also preferably between procedures.

5. “Working condition” is an established point of attention in the work-assessment meetings of the department. Co-workers are expected to offer their own points of attention concerning working conditions at these meetings. Unsafe situations must be immediately reported to their supervisors.

6. Proper disposal of clinical specimens, laboratory waste and chemical waste is of fundamental importance, for both yourself and others.

7. In the event of an accident, where staff are infected or believed to have a risk of infection, prophylactic medication is advisable. Precautions should be taken while handling fresh isolates, serum or blood from the suspected patients.

ANNEXURE VIII
## Determination of serogroup status: Agglutination results with the standard “group sera”

**Name of the isolate:**

**Source:**

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Australis</td>
<td>Australis</td>
<td>Ballico</td>
<td></td>
</tr>
<tr>
<td>2. Autumnalis</td>
<td>Rachmati</td>
<td>Rachmat</td>
<td></td>
</tr>
<tr>
<td>3. Ballum</td>
<td>Ballum</td>
<td>Mus 127</td>
<td></td>
</tr>
<tr>
<td>4. Bataviae</td>
<td>Bataviae</td>
<td>Swart</td>
<td></td>
</tr>
<tr>
<td>5. Canicola</td>
<td>Canicola</td>
<td>H. Uterecht IV</td>
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<tr>
<td>6. Celledoni</td>
<td>Cellodoni</td>
<td>Cellodoni</td>
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<tr>
<td>7. Cynoporter</td>
<td>Cynoporter</td>
<td>3522 C</td>
<td></td>
</tr>
<tr>
<td>8. Dejasiman</td>
<td>Dejasiman</td>
<td>Dejasinman</td>
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</tr>
<tr>
<td>9. Grippotyphosa</td>
<td>Grippotyphosa</td>
<td>Moskva V</td>
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<tr>
<td>10. Hebdomadis</td>
<td>Hebdomadis</td>
<td>Hebdomadis</td>
<td></td>
</tr>
<tr>
<td>11. Icterohaemorrhagiae</td>
<td>Icterohaemorrhagiae</td>
<td>RGA</td>
<td></td>
</tr>
<tr>
<td>12. Javanica</td>
<td>Poi</td>
<td>Poi</td>
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</tr>
<tr>
<td>13. Louisiana</td>
<td>Louisiana</td>
<td>LSU 1945</td>
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</tr>
<tr>
<td>14. Manhao</td>
<td>Manhao</td>
<td>L 60</td>
<td></td>
</tr>
<tr>
<td>15. Mini</td>
<td>Mini</td>
<td>Sari</td>
<td></td>
</tr>
<tr>
<td>16. Panama</td>
<td>Panama</td>
<td>CZ 214 K</td>
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</tr>
<tr>
<td>17. Pomona</td>
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<td>Pomona</td>
<td></td>
</tr>
<tr>
<td>18. Pyrogenes</td>
<td>Pyrogenes</td>
<td>Salinem</td>
<td></td>
</tr>
<tr>
<td>19. Sejroe</td>
<td>Hardjo</td>
<td>Hardjopraj</td>
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<tr>
<td>20. Shermani</td>
<td>Shermani</td>
<td>1342 K</td>
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</tr>
<tr>
<td>21. Sermin</td>
<td>Weaveri</td>
<td>CZ390</td>
<td></td>
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</tbody>
</table>
Leptospirosis

Laboratory Manual

Regional Medical Research Centre
Indian Council of Medical Research
Port Blair